Molecular Analysis of the Anaerobic-inducible Operon *nrdDG* from *Salmonella typhimurium*

by

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Abstract

Ribonucleotide reductase, which reduces ribonucleotide into deoxyribonucleotide, is an essential enzyme for DNA synthesis. Activation of this enzyme needs another small activase protein.

In this study, the nucleotide sequences of the genes encoding anaerobic ribonucleotide reductase (NrdD) and its activase (NrdG) in *S. typhimurium* were determined. *nrdD* gene contains a putative open reading frame of 2136 bp which can encode a polypeptide with 712 amino acid residues. Putative binding sequences for global regulators, Fnr and ArcAB, can be identified in the promoter region of *nrdD*. *nrdG* gene contains a putative open reading frame of 462 bp which can encode a polypeptide with 154 amino acid residues. A short intervening sequence can be found between these two genes and no promoter consensus sequence can be identified in this region. The predicted amino acid sequences of NrdD and NrdG of *S. typhimurium* show striking homology to *E. coli* anaerobic ribonucleotide reductase and its activase respectively.

Many genes important in anaerobic metabolism of enteric bacteria are under transcriptional control. The expression of *nrdD of S. typhimurium* was studied by using reverse transcriptase polymerase chain reaction (RT-PCR) as well as Northern blot analysis. By using RT-PCR, a 24-fold increase of *nrdD* transcripts amount was found in anaerobic environment. Northern blot analysis revealed a 2.4 kb signal corresponding to *nrdD* transcript that can be found only in anaerobic environment. By means of RNA dot blot analysis, the effect of Fnr on *nrdD* expression was investigated. Comparing with the wild type, the expression level of *nrdD* transcripts

was found to be decreased anaerobically in a mutant defective in Fnr while their aerobic expressions showed no change. It suggests that Fnr acts as an anaerobic activator of *nrdD* expression.

The physiological role of NrdD was also studied by comparing the growth patterns of a mutant defective in NrdD. Surprisingly, this mutant can grow equally well in both aerobic and anaerobic environments. However, a mutant defective in both NrdA (aerobic ribonucleotide reductase) and NrdD show impaired anaerobic growth. This suggests NrdA can complement the function of NrdD.

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Abbreviations

Abbreviations used in this thesis without definition include:

ATP	Adenosine triphosphate
BSA	Bovine serum albumin
bp	base pair
cDNA	Complementary DNA
CTP	Cytidine triphosphate
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycystine triphosphate
DEPC	Diethylpyrocarbonate
dGTP	Deoxyguanosine triphosphate
dTTP	Deoxythymine triphosphate
EDTA	Ethylene diamine tetraacetic acid
GTP	Guanosine triphosphate
IPTG	Isopropyl-1-thio-β-galactosidase
kb	kilo-bases
kD	kilo-dalton
MOPS	3-[N-Morpholino] propanesulfonate
PCR	Polymerase chain reaction
PEG	Polyethylglycol
PVP	Polyvinylpyrrolidone
SDS	Sodium dodecyl sulfate (Sodium lauryt sulfate)
Tris	Tris(hydroxymethyl)aminomethane
TTP	Thymine triphosphate
UTP	Uridine triphosphate
X-gal	5-bromo-4-chloro-3-indolyl-β-galactoside

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Chapter 1. General introduction.

This thesis is the report of my study on the molecular analysis of *nrdDG* operon encoding anaerobic ribonucleotide reductase and its activase from *Salmonella typhimurium* LT2. The nucleotide sequence of the *nrdDG* operon and the expression level of *nrdD* were determined. A mutant defective in *nrdD* was also characterized.

After reviewing the relevant literature in the next chapter, chapter 3 will focus on the subcloning and sequencing of the *nrdDG* operon from *S. typhimurium*. The regulation of transcription of the *nrdD* gene will be discussed in chapter 4. Characterization of a *nrdD* mutant will be described in chapter 5.

Enteric bacteria, such as *E. coli* and *S. typhimurium*, are capable of living in both aerobic and anaerobic environments. They can divert their metabolic pathways under different conditions in which different sets of genes are activated and repressed. Enteric bacteria possess ribonucleoside-triphosphate reductase for reduction of ribonucleotide anaerobically. The anaerobic enzyme is different from the one used in the aerobic environment. The main function of ribonucleotide reductase is to reduce ribonucleotides in order to make up the pool of deoxyribonucleotides, the building blocks of DNA. Three classes of ribonucleotide reductases are present in living organisms (Reichard, 1993). Class I enzymes are found in higher organisms and some prokaryotes. Class II enzymes are found solely in prokaryotes such as *Lactobacillus.* Class III ribonucleotide reductases are present in extracts of anaerobically grown *E. coli* (Fontecave *et al.*, 1989). The gene encoding this Class III enzyme was cloned, sequenced and designated as *nrdD* in *E. coli* (Sun *et al.*, 1993). In this study, a homologue of *nrdD* from *S. typhimurium* was cloned and sequenced. Downstream of

this gene was another open reading frame which showed homology of the structural gene of anaerobic ribonucleotide reductase activase, NrdG, of *E. coli*. The amino acid of these two proteins were deduced from the nucleotide sequence and compared with homologues of other organisms.

The expression of the *nrdD* gene of *S. typhimurium* was found to be up-regulated in anaerobiosis by using reverse transcriptase polymerase chain reaction (RT-PCR) and Northern blot analysis. The regulation was dependent on global regulator Fnr as revealed by RNA dot blot analysis.

S. typhimurium mutant defective in NrdD showed no impaired anaerobic growth. However, mutant with both *nrdA* and *nrdD* mutations grew poorly under anaerobic conditions.

Chapter 2. Literature review

2.1 Biosynthesis of deoxyribonucleotide triphosphates

Except in some RNA viruses, the genetic materials of most living organisms are made up of deoxyribonucleic acid (DNA). DNA composed of four different kinds of deoxyribonucleotide triphosphates (dNTPs). Biosynthesis of dNTPs in both eukaryotes and prokaryotes are largely similar. Different biochemical reactions catalyzed by several enzymes lead to the *de novo* synthesis of dNTPs (Neuhard and Kelln, 1996; Reichard, 1988).

For purine deoxyribonucleotides, dATP and dGTP, they are synthesized from reduction of corresponding ribonucleotides by ribonucleotide reductases. Aerobically, the enzyme use ribonucleotide diphosphates (ADP and GDP) as substrates. As a result, dADP and dGDP are produced and further phosphorylated into dATP and dGTP by nucleotide diphosphate kinase which is not substrate specific. In an anaerobic environment, ribonucleotide triphosphates (ATP and GTP) are directly reduced into dATP and dGTP by anaerobic ribonucleotide reductase.

For pyrimidine nucleotide biosynthesis, CDP is reduced into dCDP by ribonucleotide reductase and phosphorylated into dCTP by nucleotide diphosphate kinase aerobically while CTP is reduced directly into dCTP by anaerobic ribonucleotide reductase. The biosynthesis of dTTP, however, is much more complicated since neither TTP nor TDP is present in the cell acting as substrates for ribonucleotide reductase. Instead, UDP and UTP are used as precursors for synthesis of dTTP. In bacteria, dCTP will be transformed into dUTP by dCTP deaminase. dUTP cannot be used as building block of DNA, instead, dUTP will be firstly

dephosphorylated into dUMP by dUTPase. dUMP is then methylated into dTMP by thymidylate synthase, and dTMP is further phosphorylated into dTDP by thymidylate kinase. dTTP comes from phosphorylation of dTDP by nucleotide diphosphate kinase. dTTP can be synthesized by another route in which UDP is reduced into dUDP by ribonucleotide reductase aerobically. dUDP is transformed into dUTP by nucleotide diphosphate kinase and dUTP is further metabolised into dTTP as described above. dUTP is also synthesized anaerobically by reducing UTP by anaerobic ribonucleotide reductase. It should be noted that dCTP deaminase is absent from mammalian cells. On the other hand, dCMP deaminase is present and it will transform dCMP into dUMP. dUMP will be further metabolised into dTTP as described. dCMP is a product from dephosphorylation of dCDP by CMP kinase. The complete pathway of *de novo* synthesis dNTPs is illustrated in Fig. 2.1

A balanced supply of dNTPs to the bacterial cell is very important. It relies on a precise regulation of enzyme involved in the biosynthetic pathway. However, only two enzymes, ribonucleotide reductase and dCTP deaminase (dCMP deaminase in mammalian cells), in this pathway are under allosteric control (Neuhard and Kelln, 1996; Reichard, 1988). dCTP deaminase (dCMP deaminase) is activated by dCTP and inhibited by dTTP. Hence, its activity depends on the relative amount of dCTP and dTTP available for DNA synthesis (Neuhard and Kelln, 1996; Reichard, 1988). Ribonucleotide reductase is under a more complex allosteric control and will be discussed later.

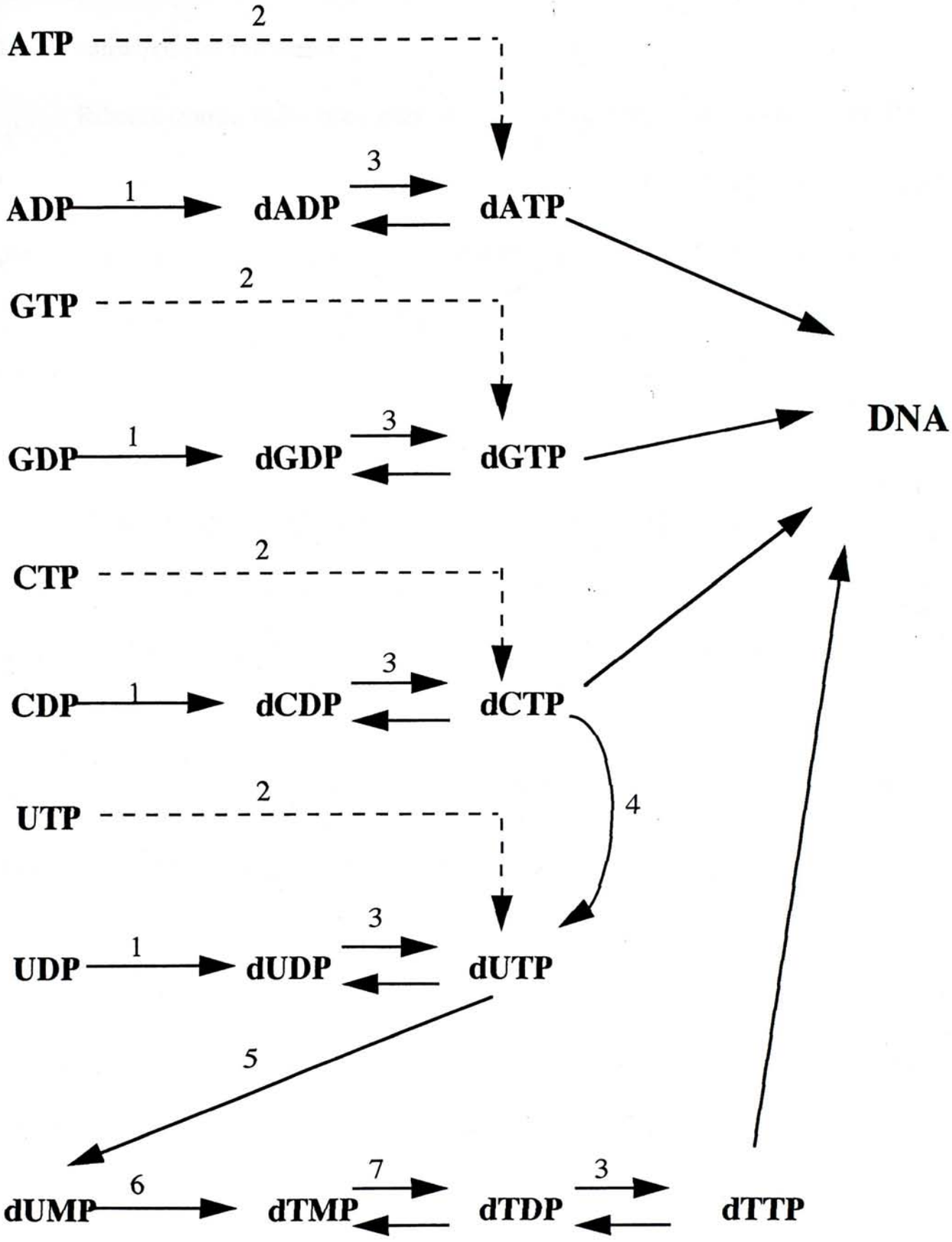


Fig.2.1 The complete pathway of de novo synthesis of dNTPs in bacteria.Enzymes:1. ribonucleotide reductase. 2. anaerobic ribonucleotide reductase. 3. nucleotide diphosphate kinase. 4. dCTP deaminase. 5. dUTPase. 6. thymidylate synthase. 7. thymidylate kinase. Broken arrows indicate reaction take place in anaerobic conditions.

2.2 Ribonucleotide reductase

Ribonucleotide reductases play a key role in the biosynthesis of dNTPs. Different organisms use different types of ribonucleotide reductases to achieve this purpose. It is generally accepted that ribonucleotide reductases can be classified into three classes (Reichard, 1993 and 1997).

2.2.1 Class I ribonucleotide reductase.

Class I ribonucleotide reductase can be found in higher organisms and some prokaryotes for which the *E. coli* enzyme is the prototype. Three dimensional (3D) structure of the *E. coli* ribonucleotide reductase has been characterized (Nordlund *et al.*, 1990; Nordlund and Eklund, 1993; Uhlin and Eklund, 1994). The enzyme is made up of two subunits α and β organized as an $\alpha_2\beta_2$ structure with a total molecular mass (M_r) of 258 kD. α_2 and β_2 are denoted as R1 and R2 respectively. R1 has a M_r of 171 kD and the R2 has a M_r of 87 kD. The schematic representation of the enzyme is illustrated in Fig. 2.2.

Several features important for ribonucleotide reduction have been characterized. The ribonucleotide reductase has a radical which is essential for the catalysis of the reaction. This radical was identified at Tyr122 of the R2 protein by site directed mutagenesis (Larsson and Sjöberg, 1986). This tyrosine radical is situated deeply inside the R2 protein (about 10 Å from the surface) as revealed by the 3D structure (Nordlund *et al.*, 1990; Nordlund and Eklund, 1993). The radical is thought to be transferred to the R1 protein by long range electron transfer to generate a thiyl radical which was identified to be located at Cys439 of the R1 protein (Mao *et*

al., 1992b). An iron centre in the R2 protein is used to generate and maintain the tyrosyl radical in the presence of oxygen. Active thiols used for reduction are all from cysteines residues of the R1 protein. Two redox active cysteine, Cys225 and Cys462, of R1 directly reduce the NDPs (Mao *et al.*, 1992a).

After the reduction, a disulfide bridge is formed between Cys225 and Cys462. The disulfide bridge is reduced into two thiols again by accepting electrons from NADPH. The electrons from NADPH first reduce thioredoxin or glutaredoxin, then, these electrons will be transferred to the disulfide bridge with two possibilities. The first one is direct electron transfer between the disulfide bridge and the reduced thioredoxin or glutaredoxin (Uhlin and Eklund, 1994). The other possibility is electron transfer via two redox active cysteine, Cys754 and Cys759, of the C-terminal of the R1 protein (Åberg *et al.*, 1989). Ribonucleotide reductase with reduced thiols and tyrosyl radical can take part in another catalytic cycle.

Class I ribonucleotide reductases are under allosteric control. Two allosteric sites are present on the R1 protein. One governs the overall activity and the other governs substrate specificity (Reichard, 1993). Cys292 of the R1 protein was found to be important for substrate specificity (Ormo and Sjöberg, 1996). The detailed mechanisms of allosteric control will be discussed later. By comparing the amino acid sequences of class I ribonucleotide reductase of different organisms, 42 invariant residues in R1 and 16 residues in R2 were determined (Sjöberg, 1995). Some of them were identified to carry specific functions for ribonucleotide reduction. They are shown in Tables 2.1a and b.

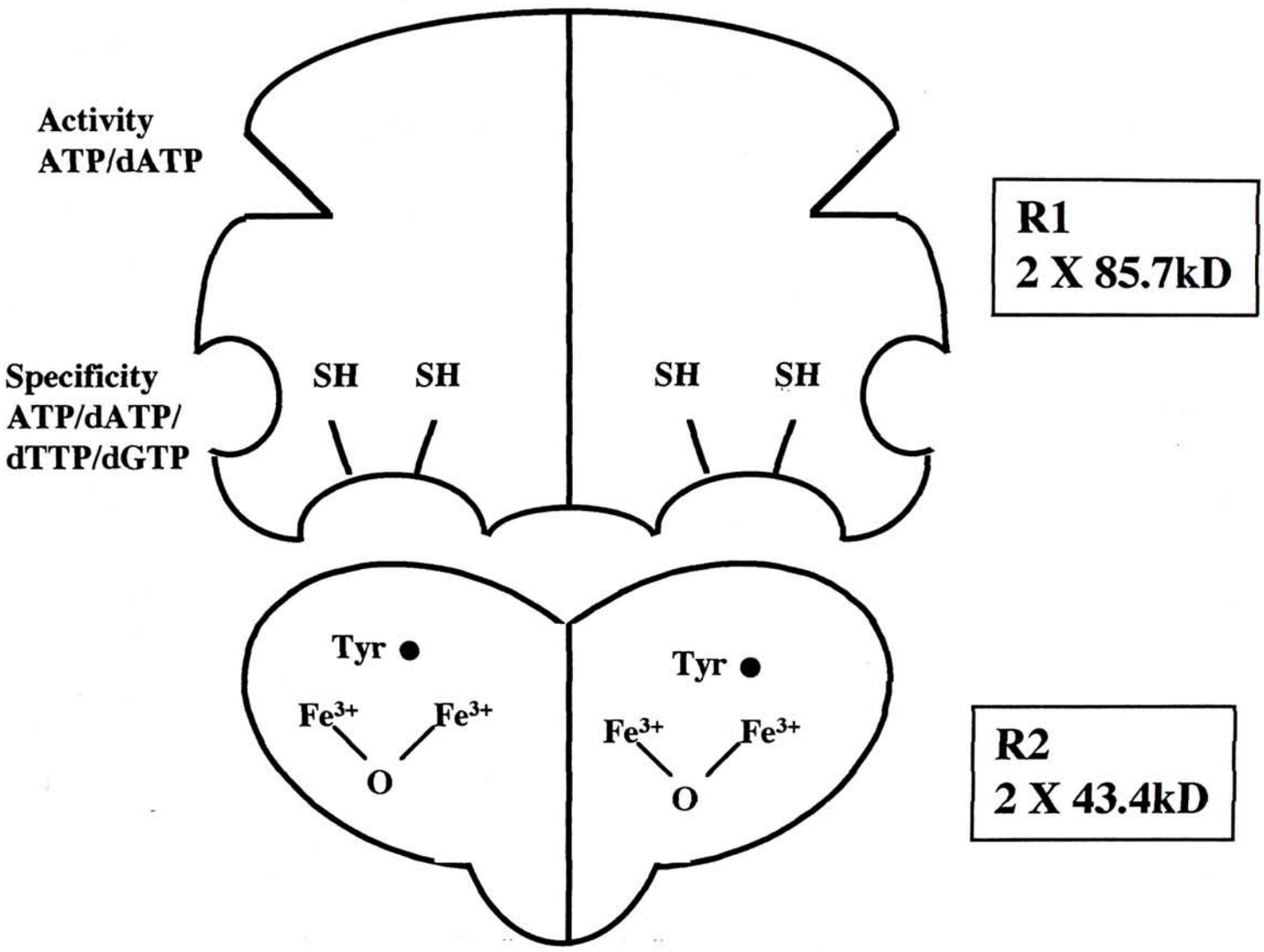


Fig.2.2 Schematic representation of *E. coli* ribonucleotide reductase. Protein R1 contains the catalytic site with its active thiols and two kinds of allosteric sites. Protein R2 has two Fe centres and tyrosyl radical. (Adopted from Reichard, 1993).

Residues	proposed functions
Pro210	Active site residue
Ser224	Active site residue
Cys225	Active site residue, redox active cysteine
Arg298	Allosteric specificity site
Arg329	R2 interaction area
Asn437	Active site residue
Leu438	Active site residue
Cys439	Active site residue, transientcysteinyl radical
Glu441	Active site residue
Cys462	Active site residue, redox active cysteine
Leu464	Active site residue
Asn613	R1-R2 interaction
Met620	Active site residue
Pro621	Active site residue
Ser692	Active site residue
Ser694	Active site residue
Tyr730	Electron transfer pathway
Tyr731	Electron transfer pathway
Cys754	Thioredoxin/glutaredoxin interaction, redox active cysteine
Cys759	Thioredoxin/glutaredoxin interaction, redox active cysteine

Table 2.1a Invariant residues in theR1 family. (Only those with defined functions are shown, modified from Sjoberg, 1995.)

Residue	Proposed functions
Trp48	Electron transfer pathway
Asp84[a]	Iron ligand, electron transfer pahtway
Glu115	Iron ligand
His118	Iron ligand, electron transfer pathway
Tyr122	Stable tyrosyl radical
Glu204	Iron ligand
Phe208	Hydrophobic pocket, radical stability
Ser211[b]	Proton transport during reduction of iron centre
Phe212	Hydrophobic pocket, radical stability
Ile234	Hydrophobic pocket, radical stability
Arg236	R1 interaction area
Asp237	Electron transfer pathway
Glu238	Iron ligand
His241	Iron ligand
Glu350	R1 interaction area, electron transfer pathway?
Tyr356	Electron transfer pathway

[a]Glutamic acid in Epstein-Barr virus sequence
[b]Histidinein African swine fever virus sequence

Table 2.1b Invariant residues in theR2 family. (Only those with defined functions are shown, modified from Sjoberg, 1995.)

The primary structure of the *E. coli* ribonucleotide reductase operon was determined (Carlson *et al*, 1984). The operon consists of two open reading frames (ORF), the first one is *nrdA* encoding R1 protein. The second ORF is *nrdB* encoding R2 protein. The *nrdAB* operon maps at the 48.5 minutes of the *E.coli* genome.

Using *lacZ* fusion, expression of *nrdA* and *nrdB* genes of *E. coli* was found to be decreased under anaerobiosis(Casado *et al.*, 1991). Under fermentative or nitrate respiring conditions, *nrdA* and *nrdB* transcribe at a lower level from the *nrdA* promoter. However, transcription of *nrdB* gene from the internal *nrdB* promoter is not affected by the absence of oxygen (Casado *et al.*, 1991). The expression of the operon is also cell cycle regulated. By investigating a synchronized culture of *E. coli*, both *nrdAB* mRNA and R2 subunit protein were found to be increased when DNA synthesis is initiated and decreased back to basal level after DNA synthesis is initiated (Sun and Fuchs, 1992). Fis and DnaA proteins bind at the promoter region of the *nrdAB* operon and have positive effects on the expression of *nrdAB* (Augustine *et al.*, 1994). But they are not required for cell cycle regulation of *nrdAB* expression. Instead, an upstream *cis*-acting AT-rich element is important for cell cycle control of *nrdAB* (Sun *et al.*, 1994).

Recently, the genes encoding a second class I of ribonucleotide reductase (NrdEF) from *S. typhimurium* were cloned and sequenced (Jordan *et al.*, 1994a). The two subunits of NrdEF analogous to NrdAB R1 and R2 proteins are designated as R1E and R2F respectively. The amino acid sequence of R1E and R2F showed overall 25 and 28% similarities to R1 and R2 proteins of different organisms. Specific

cysteine residues representing the essential thiols of the R1 and tyrosine residue of R2 important for radical chemistry are all conserved in NrdEF. A plasmid harboring these genes can complement an obligate anaerobic *E. coli nrdB*::Mud1 mutant to grow aerobically. The over-expressed NrdEF can reduce CDP into dCDP in the presence of dithiothreitol (DTT), reduced glutaredoxin. R2F contains antiferromagnetically coupled dinuclear iron centre and tyrosyl radical as NrdAB (Jordan *et al.*, 1994b). This enzyme, however, is not essential and its expression cannot be detected in *E. coli* under normal physiological conditions (Jordan *et al.*, 1996a). However, homologues of NrdEF in other organisms such as *Lactococcus lactis* and *Bacillus subtilus* are functional and essential (Jordan *et al.*, 1996b; Scotti, 1996). This second class I ribonucleotide reductase has a different allosteric control when compared to NrdAB. Moreover, it uses NrdH protein, a different electron donor, for reduction (Jordan *et al.*, 1996b).

2.2.2 Class II ribonucleotide reductase

Class II ribonucleotide reductase are found solely in prokaryotes with the *Lactobacillus leichmannii* enzyme as the prototype. The class II enzyme reduces nucleotide triphosphates instead of diphosphates. It functions both in the presence or absence of oxygen (Reichard, 1997). The *L. leichmannii* enzyme is a monomer and requires adenosylcobalamin (AdoCbl) as a cofactor (Booker and Stubbe, 1993). Radical chemistry was proposed to reduce ribonucleotide by this enzyme (Stubbe, 1989). A homolytic cleavage of the carbon-cobalt bond in AdoCbl will generate a 5'deoxyadenosyl radical and a cobalamin(II) molecules. The former molecule acts as the tyrosyl radical in the class I enzyme and generate a thiyl radical in the class II enzyme important for reduction of ribonucleotides. The amino acid residue bearing the thiyl radical was identified as Cys408 of the protein when the nucleotide sequence encoding this enzyme had been determined (Booker and Stubbe, 1993). In the same investigation, Cys731 and Cys736 were suggested to shuttle electrons from the *in vivo* reductant thioredoxin to the active-site Cys119. The overall radical chemistry, therefore, is similar to class I reductase. Allosteric control of this reductase is also similar to class I enzyme. The 3D structure as well as the regulation of expression of this enzyme have not been well characterized and will provide an interesting research areas to be explored.

2.2.3 Class III ribonucleotide reductase

Class III anaerobic ribonucleotide reductase was first isolated from anaerobically grown *E. coli* (Fontecave, 1989). The *E. coli* enzyme lose its activity upon exposure to oxygen. The components essential for this enzyme to reduce ribonucleotide triphosphates to deoxyribonucleotide triphosphates include S-adenosylmethionine, NADPH, DTT, potassium and magnesium ions (Eliasson *et al.*, 1992; Harder *et al.*, 1992). The primary structure of *E. coli* anaerobic ribonucleotide reductase was determined when the gene encoding this enzyme had been cloned and sequenced. The gene encoding this enzyme was designated as *nrdD* (Sun *et al.*, 1993). The gene encodes a peptide of 712 amino acid residues. An Fnr binding site was identified upstream of the coding sequence. The gene was mapped at the 96 minutes of the genome. The amino acid sequence of the protein predicted from *nrdD* showed homology only to the N-terminal of NrdAB. However, a striking homology was found between pyruvate formate lyase (Pfl) and NrdD at the C-terminal (Sun *et al.*, 1993). The over-expressed NrdD will be truncated at Gly681 upon exposure to oxygen (King and Reichard, 1995; Sun *et al.*, 1993). This residue was later found to form a glycyl radical when activated (Sun *et al.*, 1996). Activation of NrdD needs another protein NrdG encoded by an open reading frame downstream of *nrdD* (Sun *et al.*, 1995). An Fe-S cluster was first proposed to occur on the NrdD protein (Mulliez *et al.*, 1993). However, the Fe-S cluster was later found to be situated in NrdG (Ollagnier *et al.*, 1996). The misinterpretation was due to contamination of NrdG in the preparation of NrdD in the study. NrdD and NrdG were proposed to form a tight complex and exist as an $\alpha_2\beta_2$ structure analogous to R1 and R2 protein of NrdAB

(Ollagnier *et al.*, 1996). Presence of this small activase (NrdG) and glycyl radical suggests that anaerobic ribonucleotide reductase is very similar to Pfl. It was proposed that S-adenosylmethionine interact with NrdG to generate a transient radical in the protein similar to that of the Pfl activase. The radical is then transferred to the Gly681 of NrdD and participate in the catalytic process via a transient thiyl radical (Ollagnier *et al.*, 1996).

Instead of using thioredoxin or glutaredoxin as reductant, NrdDG proteins use formate as electron donor for ribonucleotide reduction (Mulliez *et al.*, 1995). Hydrogen in the form of tritium from formate was found to be transferred to water but not to the dNTPs but hydrogen from water in form of deuterium was found in dNTPs during ribonucleotide reduction performed by NrdDG (Eliasson *et al.*, 1995). However, cysteine residues carrying the thiols for reduction have not been identified in NrdDG.

Either NrdD or NrdG is essential for anaerobic growth of *E. coli*. Knock-out mutants in either *nrdD* or *nrdG* were unable to survive in strict anaerobic environments (Garriga *et al.*, 1996). However, in microaerophilic environments, these mutants can grow well due to increased expression of aerobic ribonucleotide reductase NrdAB. Information about the structural properties of NrdDG is scarce. The regulation of transcription of *nrdD* or *nrdG* has not yet been well studied although Fnr was suggested to be important in *E. coli nrdD* expression (Sun *et al.*, 1993). But direct evidences such as quantification of transcripts or data from operon fusion are absent. However, the expression of *nrdD* was found to be increased 8-fold under

anaerobiosis by fusing the promoter region of *S. typhimurium nrdD* to the *lacZ* reporter gene on a promoter probe plasmid pFZY1 (Wong, 1990).

nrdAB and *nrdEF* genes were cloned and sequenced in *S. typhimurium*, a phylogenetically related organism to *E. coli* (Jordan *et al.*, 1994a and 1995). But only the 5' untranslated region of the *nrdDG* genes has been characterized in this organism (Wong, 1990). Bacteriophage T4 possess a homologous sequence to *nrdDG* which encode anaerobic ribonucleotide reductase and its activase for anaerobic infection of *E .coli* (Young *et al.*, 1994 a and b, 1996).

2.3 Proposed mechanism for ribonucleotide reduction

There is only little sequence homology among three classes of ribonucleotide reductases, and the cofactors involved in the catalysis are different. However, all these enzymes were proposed to use the same radical chemistry to reduce ribonucleotides (Reichard, 1993 and 1997; Sjöberg, 1995; Stubbe, 1989). Generally, four important characteristics are essential for ribonucleotide reduction: a protein radical, radical generator, redox active cysteines and a reductant.

The reduction mechanism could not be ascertained until the 3D structure of the R1 and R2 protein of class I ribonucleotide reductase had been characterized (Norlund *et al.*, 1990; Nordlund and Eklund, 1993; Uhlin and Eklund, 1994).

Briefly, a protein radical is generated in the reductase by different radical generators. In class I *E. coli* enzyme, the radical is generated at tyr122 of the R2 protein by means of an iron centre in the presence of oxygen. In class II *L. leichmannii* enzyme, the radical is generated by interaction with a homolytic cleaved adenosylcobalamin. In class III anaerobic *E. coli* enzyme, a glycyl radical is produced by S-adenosylmethionine via an activating enzyme which carries a Fe-S cluster. The radical, especially the tyrosyl radical in the R2 protein, was originally thought to abstract a hydrogen atom from the 3'-position of the ribose moiety of the ribonucleotide (Stubbe, 1989). However, as revealed by the 3D structure, the position of this tyrosyl radical was found to be located deeply inside the protein and cannot easily access the ribonucleotide. Long-range electron transfer from the radical was proposed to generate a thiyl radical at Cys439 in the R1 protein (Norlund *et al.*, 1990; Nordlund and Eklund, 1993). An analogous Cys408 in the *L. leichmannii* enzyme was

identified to be responsible for this purpose (Booker and Stubbe, 1993). Several amino acids residues important for this electron transport pathway were identified in the R1 and R2 proteins (Sjöberg, 1995). The thiyl radical will abstract a hydrogen atom from the ribose moiety of the ribonucleotide to form a 3'-ribonucleotide radical. Subsequently, a hydrogen atom from a thiol of a redox active cysteine is added to the 2'-hydroxyl of ribonucleotide radical. A cation radical intermediate is generated by loss of a water molecule. This intermediate abstracts another hydrogen atom from the thiol of another redox active cysteine. The two oxidized thiols will then form a disulfide bridge. Redox active cysteine residues were identified as Cys225 and Cys462 in the R1 protein. These redox active cysteine residues were also identified as Cys119 and Cys419 in *L. leichmannii* enzyme. Finally, the radical intermediate abstracts the hydrogen atom from the reduced thiyl radical to form the dNTP. The thiyl radical is hence regenerated. The proposed mechanism for ribonucleotide reduction is schematically represented as Fig. 2.3.

The disulfide bridge has to be reduced first for the reductase to take part in another cycle of catalysis. In class I and class II enzymes, NADPH donates its electron to thioredoxin or glutaredoxin. By transthiolation from the reduced thioredoxin and glutaredoxin to the reductase, the disulfide bridge is reduced. However, in class III anaerobic enzyme, formate is used to reduce the disulfide bridge. After the disulfide bridge has been reduced, the reductase can participate in another cycle of catalysis. Table 2.2 presents a comparison of characteristics of different classes of ribonucleotide reductases.

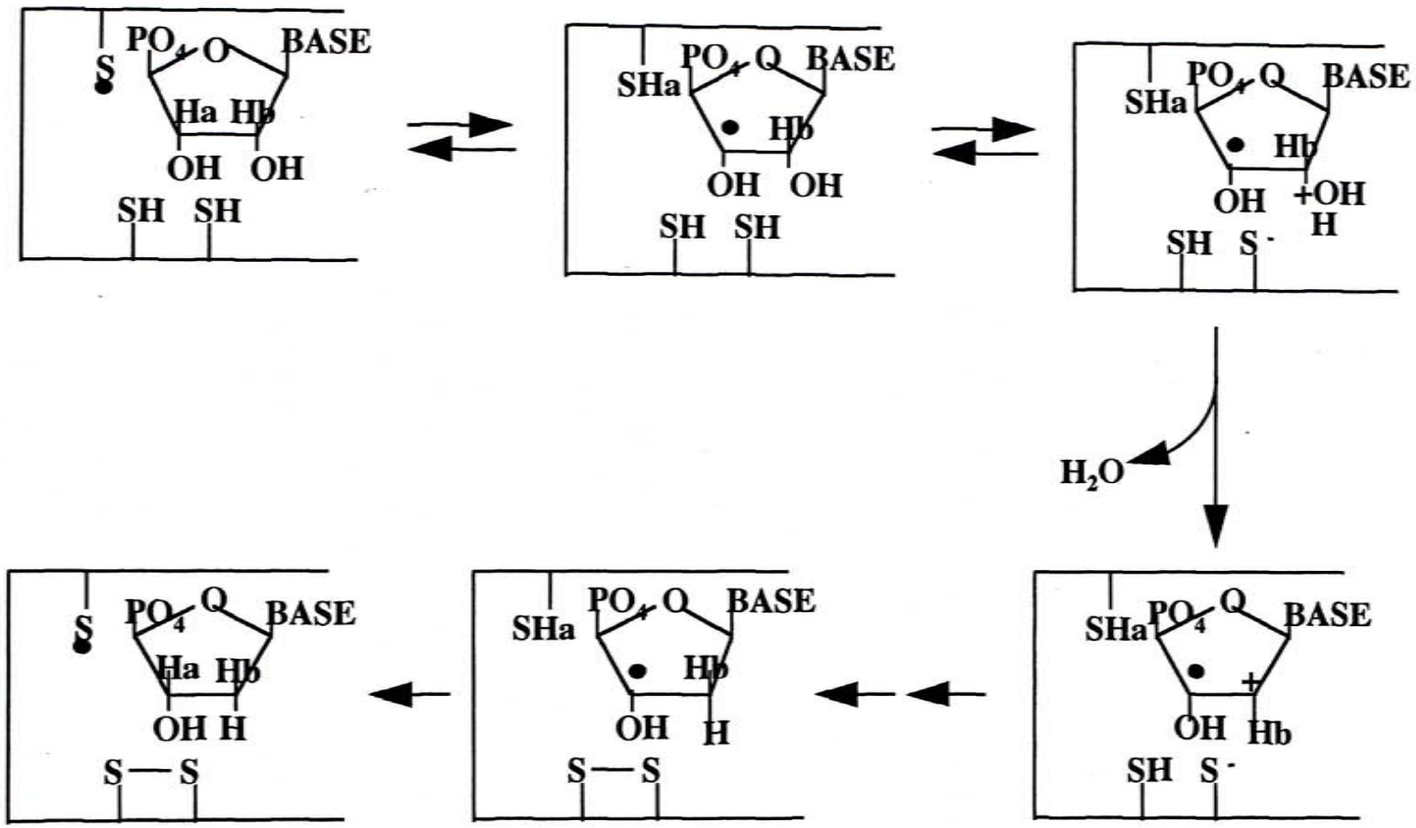


Fig. 2.3 Schematic representation of proposed mechanism for ribonucleotide reduction. (Modified from Stubbe,1988)

	Class I		Class II	Class III
Respiration	aerobic	aerobic	aerobic/anaerobic	anaerobic
Occurrence	*E. coli*/eukaryotes	bacteria	bacteria	bacteria
Structure	$\alpha_2\beta_2$	$\alpha_2\beta_2$	$\alpha(\alpha_2)$	$\alpha_2\beta_2$
Metal	Fe-O-Fe	Fe-O-Fe	Co	4Fe-4S
Radical	Tyr	Tyr	adenosylcobalamin	adenosylmethionine
reductant	thioredoxin/glutaredoxin	NrdH	thioredoxin/glutaredoxin	formate
dATP	inhibitory	stimulatory	stimulatory	stimulatory

Table 2.2 Comparison of different classes of ribonucleotide reductases. (Modified from Reichard, 1997).

2.4 Allosteric control of ribonucleotide reductase

Another important feature common to three classes of ribonucleotide reductases is that a single enzyme can reduce each of the four ribonucleotides. A balance supply of each ribonucleotide is governed by a sophisticated allosteric control mechanism. The specificity of substrate being reduced is controlled by binding an effector molecule to a specific site of the enzyme distinct from the catalytic site. One of the effector molecules (ATP, dATP, dGTP or dTTP) binds to the enzyme and modify the tertiary structure of the catalytic site in order to reduce a suitable substrate. An additional activity site is present in class I *E. coli* and eukaryotic R1 protein to regulate the overall activity of the reductase independently of its specificity. Binding of ATP or dATP at this site acts as a stimulator or an inhibitor respectively.

2.4.1 Allosteric control of class I ribonucleotide reductase.

As discussed above, two different forms of class I reductase, NrdAB and NrdEF, have been identified. The allosteric controls of these two forms of enzyme are slightly different.

The allosteric control of the *E. coli* NrdAB is most well studied (Reichard, 1993). The protein is composed of two homodimers R1 and R2. The two active sites controlling overall activity and specificity are both located on the R1 protein as seen in Fig 2.2. The catalytic site is located also in the R1 protein and the substrates are four different kinds of nucleotide diphosphates. When the activity site binds an ATP molecule, the enzyme is active. When the activity site binds a dATP molecule, the

enzyme becomes inactive. ATP, dATP, dTTP and dGTP will bind to the substrate specificity site. The effect of accumulation of dCTPs is exert on dCMP/dCTP deaminase as discussed before. When ATP is bound on activity site of the enzyme, binding of another ATP molecule on the specificity site will reduce CDP and UDP. Binding of dTTP to the active site will reduce GDP. ADP is reduced when dGTP is bound. When the activity site as well as the substrate specific site are bound with dATP, the enzyme becomes inactive. This allosteric control contributes a physiological significance. In an actively dividing cell, the ATP level should be high and can occupy both activity and specificity sites and it renders the enzyme to reduce pyrimidine nucleotide. When pyrimidine nucleotide TTP content is high enough in the cell, it will compete the substrate specificity site with ATP and cause the enzyme to reduce GDP. When dGTP content is high enough to compete for the substrate site, the enzyme will reduce ADP. Once dATP content is high enough to compete for both activity site and specificity site, the enzyme activity is down-regulated and production of dNTP is ceased. The competition of these effectors to the activity and the specificity site ensure that a balanced supply of dNTPs can be achieved (Sjöberg, 1995).

Another class I enzyme, *S. typhimurium* NrdEF, has similar allosteric control mechanism with NrdAB enzyme. With ATP bound to the enzyme, it reduces CDP and UDP. With dTTP bound, the enzyme reduces GDP. ADP is reduced when dCTP is bound (Eliasson *et al.*, 1996). One major difference found between NrdAB and NrdEF is the effect of dATP. In high concentration (1 μM) of dATP, NrdAB activity is inhibited. However, in this concentration, NrdEF can be stimulated to

reduce CDP and UDP (Sjöberg, 1995). The difference was due to the difference in the N-terminal sequence between these two proteins. Fifty to sixty amino acids residues are missing from the N-terminal of NrdEF. This domain is responsible for binding of ATP effector which governs the overall activity of NrdAB (Sjöberg, 1995). While there is no overall activity control, a balance supply of dNTPs is maintained by higher binding affinity of dNTPs comparing with ATP (Sjöberg, 1995).

2.4.2 allosteric control of class II and class III ribonucleotide reductases.

Class II *L. leichmannii* ribonucleotide reductase has only one specificity site on the protein for the binding of dNTPs (Reichard, 1993). The enzyme reduces ribonucleotide triphosphates and the products of the reaction acts as allosteric effectors directly. Reduction of CTP is induced by dATP (and to a lesser extent ATP). UTP reduction is stimulated by dCTP, ATP reduction is stimulated by dGTP, and GTP reduction is stimulated by dTTP. The allosteric control is apparently similar to that of class I enzyme except dCTP can exert its effect on the regulation. The interaction and the competition among the four dNTPs provide a balanced pool of nucleotides to the bacteria.

Class III anaerobic *E. coli* ribonucleotide reductase, NrdDG, can reduce four different kinds of ribonucleoside triphosphate. It also shows allosteric control over the reduction process by binding specific effector molecules. However, the binding properties is slightly different from both class I and class II enzymes. In the large subunit of NrdDG, two separate binding sites for effectors were suggested (Eliasson *et al.*, 1994). The first one binds dATP or ATP while the second one binds dATP,

dGTP and TTP. When ATP binds to the first site, reduction of the two pyrimidine ribonucleotides CTP and UTP occurs. This site has ability to bind dATP also, in this situation, the enzyme lose its activity. The second site regulates the reduction of ATP and GTP. With dGTP bound, ATP is reduced. With dTTP bound, the enzyme reduces GTP. Binding of dATP to this 'purine site' results in an inactive enzyme (Eliasson *et al.*, 1994). Therefore, the aerobic and the anaerobic enzymes are different only in the ATP binding properties. NrdAB has two sites for ATP binding while NrdDG has only one. But this difference does not alter the physiological significance of allosteric control. Both enzymes reduce pyrimidine nucleotides when ATP is bound. Subsequently, binding of dTTP results in production of dGTP. Binding of dGTP leads to accumulation of dATP which in turns inactivate both reductases (Eliasson *et al.*, 1994). Thus, a balanced supply of four dNTPs can be achieved by sophisticated allosteric controls of ribonucleotide reduction in both aerobic and anaerobic environments.

2.5 Evolution of ribonucleotide reductase

Ribonucleotide reductases are ubiquitous in different organisms from archaebacteria to mammals (Harder, 1993; Reichard, 1997). Three different classes of ribonucleotide reductase are grouped by their primary structures and the cofactor used for the reaction (Reichard, 1993). Despite their differences, they share similarity on the radical chemistry used for reduction as well as allosteric control by dNTPs. Moreover, five cysteines participating in catalysis and the surrounding amino acid residues have limited homology between class I *E. coli* and class II *L. leichmannii* enzymes. It was thus suggested that these two classes are related (Reichard, 1997). The relationship of class III anaerobic enzyme with the other two classes is reflected by the sophisticated allosteric control by dNTPs in ribonucleotide reduction. Although the 3D structure was revealed in class I enzyme only, it is believed that only closely related tertiary structures can give the high degree of similarity in this aspect (Reichard, 1997). Thus, these three classes of enzymes were proposed to arise from a common ancestor through divergent evolution (Reichard, 1993).

If these three classes of ribonucleotide reductases arose from a common ancestor, what was the ancient reductase? Class II enzymes require only one polypeptide chain for catalysis and it can function in both aerobic and anaerobic environments. Adenosylcobalamin, which is prebiotic in origin, is used for generation of radical suggesting this class of enzyme resembles the ancient reductase. However, class III enzymes operate anaerobically that favor ancient environments where oxygen was limited. The enzymes use adenosylmethionine which is simpler than adenosylcobalamin. The former is considered to be an evolutionary forerunner

(Reichard, 1997). The Fe-S cluster inside the anaerobic reductase is favorable in ancient environments as minerals of sulfur-linked iron were abundant at the surface of the early Earth. On the contrary, cobalt, present in cobalamin was rare at that time (Reichard, 1997). Furthermore, one of the simplest organic reductant formate rather than complex protein reductant is used as electron donor for the anaerobic class III reductase suggesting class III reductase is close to the ancestor protein.

The proposition of class III enzyme to be the ancient reductase was challenged when the genes encoding two ribonucleotide reductases from archaebacteria *Pyrococcus furiosus* and *Thermoplasma acidophila* were cloned recently (Riera *et al*, 1997; Tauer and Benner, 1997). It was argued that nonstructural properties of proteins cannot be used reliably as indicators of homology since nonstructural features of proteins can diverge even in a pair of homologous proteins (Tauer and Benner, 1997). For ribonucleotide reductases, the choice of cofactor to generate radical in the protein appears to have diverged to reflect the availability of the cofactor in the environments. Thus, an adaptive model rather than an evolutionary model can explain the choice of cofactors (Tauer and Benner, 1997).

Secondary structure was used to predict the ancient reductase. The gene encoding *T. acidophila* B_{12}(cobalamin)-dependent ribonucleotide reductase was cloned and its secondary structure was inferred from the amino acid sequence predicted from the nucleotide sequence. When the secondary structure of this reductase was compared with other classes of reductases, it shows homology with class III anaerobic reductase at the N-terminal. Moreover, by using predicted secondary structure of *Mycobacterium tuberculosis* B12-dependent ribonucleotide reductase as 'sequence

bridge', the *T. acidophila* enzyme can form a link between the class I *E. coli* Fe-dependent and class II *L. leichmannii* B_{12}-dependent enzymes (Tauer and Benner, 1997). Similarly, the amino acid sequence of the ribonucleotide reductase from the hyperthermophilic archaebacterium *P. furiosus* show striking homology to all three different classes of reductases. Based on the cofactors used, the hyperthermophilic enzyme is defined as class II enzyme (Riera *et al.*, 1997). If protein sequence is a better criteria than nonstructural properties for determining homology, the ribonucleotide reductases from these two archaebacteria should represent the ancestor protein as these two enzymes show homology to all three classes of ribonucleotide reductases.

2.6 Central metabolic pathways of enteric bacteria

Enteric bacteria, such as *Escherichia coli* and *Salmonella typhimurium*, can grow under both aerobic and anaerobic conditions.. In aerobic environment, they derive their energy by aerobic respiration in which glucose is metabolized into pyruvate by glycolytic pathway. Pyruvate is subsequently oxidatively decarboxylated by pyruvate dehydrogenase (PDH) complex into acetyl CoA and CO_2. The resulting acetyl unit is totally oxidized in the citric acid cycle producing reducing equivalents. These reducing equivalents are transferred to oxygen via the aerobic electron transport chain composed of primary dehydrogenase, ubiquinone and cytochrome *o* or cytochrome *d* as terminal oxidases.

On the contrary, anaerobic respiration or fermentation is used in anaerobic environment. The glycolytic conversion of glucose to pyruvate is common to the aerobic respiration. Afterwards, the metabolic pathway diverts. Under anaerobic conditions, the PDH complex is repressed. Pyruvate is metabolized into formate and acetyl CoA instead by pyruvate formate lyase (Pfl). Moreover, the citric acid cycle is converted into a branched and non-cyclic form with one oxidative and one reductive route. The change is due to repression of 2-oxoglutarate dehydrogenase complex, succinyl-CoA synthetase, fumarase A and succinate dehydrogenase with induction of fumarase reductase and fumarase B by anaerobiosis. In the absence of exogenous electron acceptor, fermentation is carried out in which redox balance is achieved internally and energy is derived from substrate level phosphorylation. Acetyl CoA produced from pyruvate can be used to generate ATP from ADP by conversion to acetate, or to dispose of extra reducing equivalent by conversion to acetate. The

former reaction is catalyzed by phosphotransacetylase and acetate kinase while the latter one is catalyzed by ethanol dehydrogenase. Pyruvate is also metabolized to lactate by lactate dehydrogenase at the expense of NADH. Formate is decomposed into CO_2 and H_2 by formate hydrogen lyase. Succinate is produced in the reductive route of the branched citric acid cycle catalyzed by fumarate reductase. In this reaction, fumarate accepts electrons from internal donors via primary dehydrogenase and menaquinone acting as redox adapter. Hence, succinate acts as the electron sink in fermentation pathway.

However, when exogenous electron acceptor such as nitrate, nitrite, fumarate, trimethylamine N-oxide (TMAO) or dimethylsulfoxide is present, electrons from reducing equivalents are passed to these acceptors through an electron transport chain composed of primary dehydrogenases, fumarate reductase, and ubiquinone or menaquinone. Different enzymes such as nitrate reductase, nitrite reductase, fumarate reductase or TMAO reductase are used as terminal oxidases depending on the available electron acceptors. The central metabolic pathways under aerobic respiration, anaerobic respiration and fermentation are illustrated in Fig. 2.4a-c.

Respiration is much more favourable than fermentation when exogenous electron donors are available. Moreover, enteric bacteria preferentially use electron donor which has a higher midpoint redox potential. For instance, oxygen is favoured over nitrate, while nitrate is favoured over fumarate. This preferential choice allows the bacterial cell obtains maximum yield of energy (Lin and Iuchi, 1991).

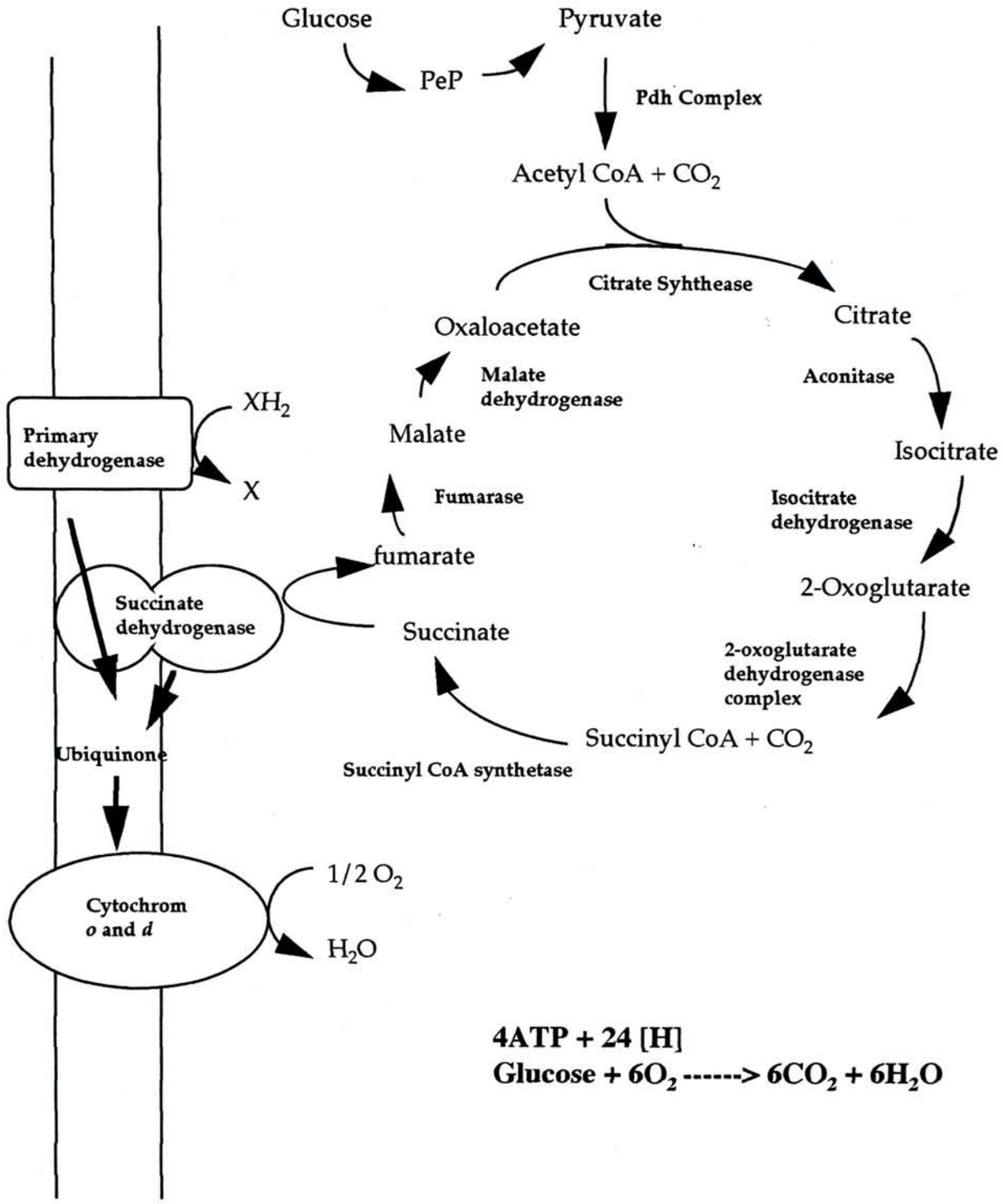


Fig2.4a The central pathways of carbon and energy metabolism of enteric bacteria under aerobic condtions (Modified from Guest, 1995).

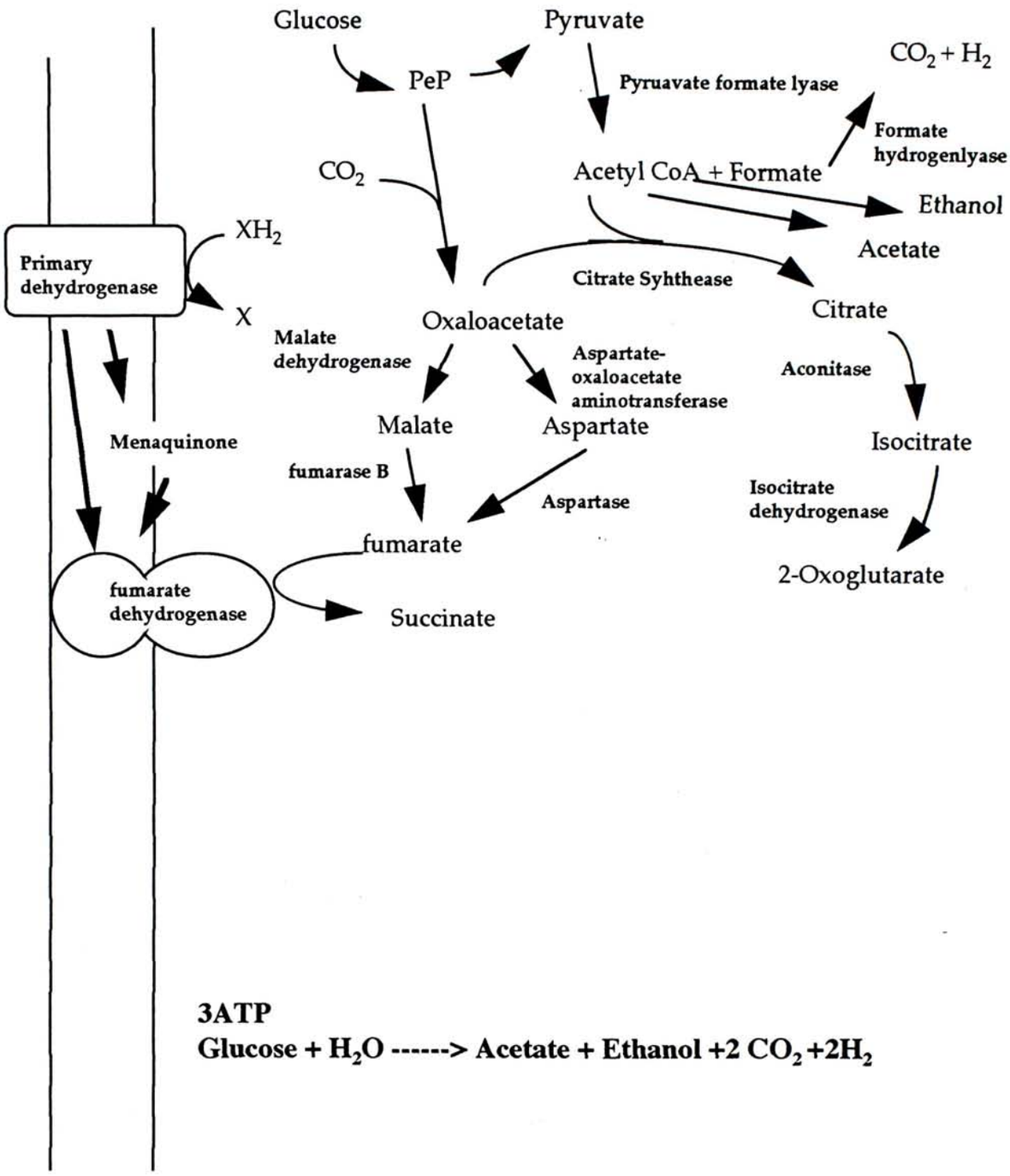
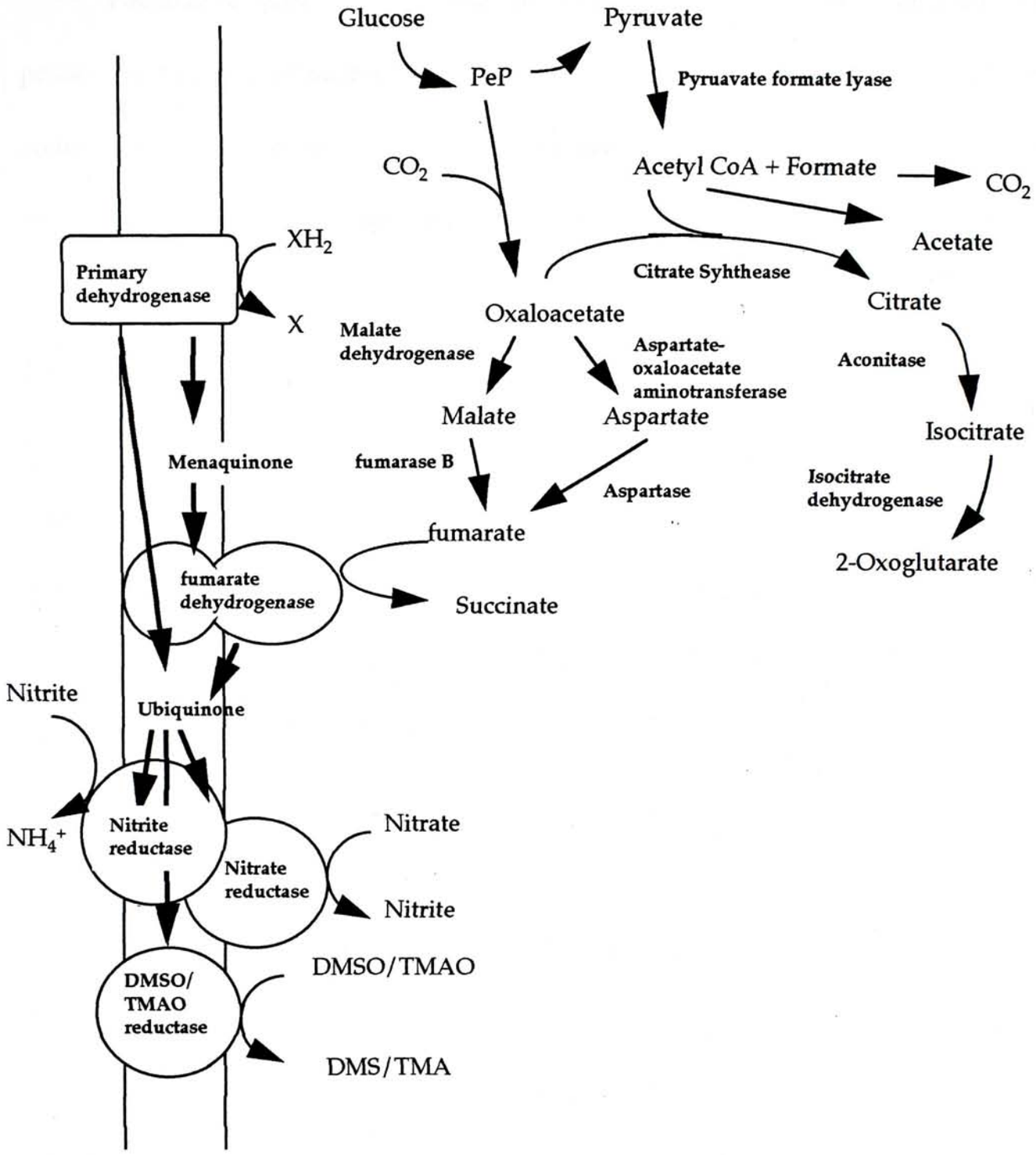


Fig2.4c The central pathways of carbon and energy metabolism of enteric bacteria under anaerobic condtions in the absence of exogenous electron acceptor (Modified from Guest, 1995).



4ATP + 8[H]

Glucose + $4NO_3^-$ ------> 2 acetate + $2CO_2$ + $2H_2O$ + $4NO_2^-$

Glucose + 4fumarate------> 2 acetate + $2CO_2$ + $2H_2O$ + 4succinate

Fig2.4b The central pathways of carbon and energy metabolism of enteric bacteria under anaerobic condtions in the presence of exogenous electron acceptor (Modified from Guest, 1995).

2.7 Regulation of gene expression by oxygen in enteric bacteria

Facultative anaerobic lifestyle of enteric bacteria is accomplished by possessing two sets of metabolic systems as discussed above. Change of metabolic context needs a coordinated regulation of various enzymes. The regulation is found to be at transcription level in response to oxygen or alternative electron acceptors. Most of the genes involved in anaerobic metabolism are grouped as modulon (Lin and Iuchi, 1991) that a family of target operons is under control of a single regulator. To date, various regulators are identified to adjust the metabolic context in order to ensure the bacteria can perform the most energetically favourable process in a given environment. The most extensively studied candidates are Fnr, ArcAB, NarXL and NarQP (Reviewed by Darwin and Stewart, 1996; Guest, 1995; Guest *et al.*, 1996; Lin and Iuchi, 1991; Lynch and Lin, 1996a; Unden *et al.*, 1995).

2.7.1 Regulation of gene expression by Fnr

Fnr was first identified in a *E. coli* mutant lacking the ability to reduce fumarate and nitrate (Lambden and Guest, 1976). The homologue in *S. typhimurium* was identified in a mutant incapable of using nitrate as electron acceptor. The mutation was designated as *oxrA* (Strauch *et al.*, 1985). Both of them were mapped at around 29 minutes of the bacterial genomes. It was later found that the Fnr protein is a transcriptional regulator of a number of operons. This protein is synthesized both under aerobic and anaerobic conditions but become active in anaerobic environment only (Guest *et al.*, 1990).

Numerous operons concerned with anaerobic metabolism are regulated by Fnr and designated as the Fnr modulon. The Fnr modulon contains 29 known transcriptional units (70 genes) in *E. coli* (Guest *et al.*, 1996). Several operons such as *pepT* (Strauch *et al.*, 1985) and *pfl* (Wong *et al.*, 1989) are regulated by *oxrA* in *S. typhimurium*. Candidates of the Fnr modulon are listed in Table 2.3. Fnr, which exists as a dimer, exerts its effect by binding to the promoter regions of the operon being regulated with consensus sequence of dyad symmetry TTGAT----ATCAA (Eiglmeier *et al.*, 1989). A good -10 promoter consensus sequence is usually found in a Fnr-activated promoter while the -35 consensus sequence is replaced by a Fnr recognition sequence at about -41.5 (Guest at al., 1996). More than one binding site can usually be found in Fnr-repressed promoter. Although there is no common pattern of organization in these promoters, deletion of the furthest upstream binding sequence severely impairs Fnr-mediated repression (Guest *et al.*, 1996).

Fnr contains several cysteine residues at the N-terminal, which are important for Fe binding to form a redox-sensitive Fe-S centre. Several mechanisms were proposed to activate the Fnr protein. Reduction of the cysteine-bound iron cofactor may convert Fnr into the active anaerobic conformation. On the other hand, Fnr may also be activated or inactivated by reversible uptake and release of ferrous iron. Other potential mechanisms such as monomer-dimer transition and interaction between Fnr and unidentified redox-signaling coeffector were also proposed (Guest, 1995; Guest *et al.*, 1996).

By using an oxystat, the expression pattern of Fnr-dependent gene was studied as a function of oxygen tension (pO_2) (Becker *et al.*, 1996). It was found that

the pO_2 values of the half-maximal repression ($pO_{0.5}$) depend on particular promoter and range from 1 to 5 millibars. And the intracellular oxygen content could provide the signal without the need for a signal transfer chain. However, there is no indication for a direct interaction between Fnr and molecular oxygen (Becker *et al.*, 1996).

Table 2.3 The Fnr modulon members in *E. coli* (Modified from Guest *et al.*, 1996)

Genes	Enzyme	Fnr site	Evidence	Other regulators
A. Fnr-activated				
aeg-46.5	Putative periplasmic nitrate reductase	-64.5	DEF	NarL(-) NarP(+)
ansB	L-asparaginase II	-41.5, -74.5?	CDEF	CAP(+)
arcA	ArcA	-82.5	DEF	ArcA(+)
aspA-dcuA	L-Aspartate and dicarboxylate transport	ND	D	NarL(-) CAP(+)
cea	Colicin E1	-64.5 to 31.5 (5 potential sites)	EF	LexA(-) CAP (+)
[cydAB]	Cytochrome *d*	-54.5	DEF	ArcA(+)
dcuB-fumB	Dicarboxylate transport and fumarase B	ND	DEF	NarL(-)
dmsABC	DMSO reductase	-49.5	DEF	NarL(-)
fdnGHI	Formate dehydrogenase-N	-42.5,-97.5	BCDEF	NarL, P (+) ArcA (-)
feoAB	Iron (II)transport	ND	DEF	Fur(-)
FFpmelR	Semi-synthetic fusion	-41.5	ABCDEF	
focA-pfl	Formate transport and pyruvate formate-lyase	P6 -41.5, (-380.5) P7 -59.5	BCDEF	ArcA(+) NarL (-) IHF
frdABCD	Fumarate reductase	-45.5 or -46.5	DEF	NarL(-)
glpABC	Anaerobic glycerol-3-P dehydrogenase	-40.5	D	NarL?

glpTQ	Glycerol 3-P transport	-91.5	D	NarL? CAP(+)
hypBCDE-fhlA	Hydrogenase activities and formate regulation	-42.5	EF	RpoN
narGHJI	nitrate reductase	-41.5	BCDEF	NarL(+) IHF
narK	nitrite extrusion protein	-41.5,-79.5	CDEF	NarL(+) IHF Fis
nikA-E(hydC)	nickel transport	ND	DEF	
nirBCD	NADH-dependent nitrite reductase	-41.5,-81.5?	BCE	NarL(+)
nrfABCDEFG	Formate-linked nitrite reductase (cyt c552)	-42.5	DEF	NarL(-) NarP(+)
nrdD	Anaerobic ribonucleotide reductase	ND	EF	
	molybdate reductase	ND	E	
B. Fnr-repressed				
[cyoABCDE]	Cytochrome *o*	ND	DE	ArcA(-) Cap(-)
fnr	Fnr	-0.5, -103.5	ABCDE	
hemA	Glutamyl-tRNA dehydrogenase	-23.5	D	ArcA(+) IHF
narX	NarX	-106.5,-75.5, +107.5	ADF	NarL(+)

ndh	NADH dehydrogenase II	-50.5,-94.5	ABDEF	RpoN
[pdhR-aceEf-lpd]	Pyruvate dehydrogenase complex and regulator	-49.5	F	ArcA?
[sodA]	Mn-Superoxide dismutase	-35.5 or -32.5	DEF	SoxRS,Q(+) Fur (-) ArcA(-) IHF

Members are listed alphabetically. The position of Fnr-site centers are defined relative to the transcriptional start points. Evidence is classified as A: *in vitro* transcription; B: *in vitro* footprinting; C: mutational analysis; D: *LacZ* fusion analysis; E: altered expression in fnr mutant; F: predicted from nucleotide sequence. ND: not determined. Uncertain member are enclosed by square blankets. The effect of other regulator is indicated as: positive (+) and negative (-) .

2.7.2 Regulation of gene expression by ArcAB

While Fnr mainly acts as an anaerobic activator, ArcAB behaves mainly as aerobic repressor. Two classes of *E. coli* mutant were identified that failed to repress succinate dehydrogenase anaerobically (Iuchi and Lin, 1988; Iuchi *et al.*, 1990). Genetic mapping of the mutations identified two regulatory loci: *arcA* at the minute 0 region of the genome and *arcB* at minute 69.5. The symbol *arc* means aerobic respiration control.

arcA was mapped at the same position of *dye* gene. Both mutants have same phenotypes which are sensitive to toluidine blue and resistance to infection by male-specific M13 phage. Hence, *arcA* was proposed to be allelic to *dye*. The nucleotide sequence of *dye* gene had been determined. It was proposed that ArcA is a pleiotropic regulator involved in mediating cellular adaptations to anaerobiosis. A high homology was found between ArcA and a class of proteins referred as 'response regulator'. Transcriptional regulations of *arcA* are mediated by Fnr and *arcA* itself (Compan and Touati, 1994; Tam, 1993).

arcB was identified in another class of mutant lacking the ability of repressing aerobic enzyme under anaerobic conditions. The nucleotide sequence of *arcB* was determined and found to be able to encode a protein homologous to 'H-box' domain of a family of proteins that serve as the sensor components of two-component signal transduction system.

The regulatroy mechanisms of ArcA-ArcB two component system was summarized recently (Iuchi and Weiner, 1996). It was proposed that ArcAB belongs to the family of two-component regulatory system in which the ArcB sensor detects

an environmental signal and modifies the activity of a response-regulator ArcA. ArcB signals the information to ArcA by transphosphorylation. ArcA-P then binds to the promoters and regulates the expressions of target operons. Disruption of *arcB* gene completely abolishes the regulatory effect of ArcAB system in response to oxygen, thus, the recognition of environmental change is totally dependent on ArcB sensor protein. It was suggested that fermentation products rather than oxygen or redox reaction involving the cysteine residues of ArcB act as the signals activating the Arc system..

A number of operons are regulated by the ArcAB system (Arc modulon) and summarized in Table 2.4. Recently, the consensus binding sequence of phosphorylated ArcA was identified (Lynch and Lin, 1996b). It is a 10 bp consensus sequence [A/T]GTTAATTA[A/T].

Table 2.4 The Arc modulon (modified from Lynch and Lin, 1996a)

Gene	Enzyme	Gene	Enzyme
ArcA-activated		**ArcA-repressed**	
arcA	ArcA	*aceB*	Isocitrate lyase
cob	Cobalamin biosynthesis	*acn*	Aconitase
cydAB	Cytochrome *d* oxidase	*cyoABCDE*	Cytochrome *o* oxidase
focA-pfl	Pyruvate formate lyase	*fadB*	3-hydroxyacyl CoA dehydrogenase
hemA	Glutamyl-tRNA dehydrogenase	*fdnGHI*	Formate dehydrogenase N
hyaA-F	Hydrogenase 1	*fumA*	Fumarase A (aerobic)
pdu	Propanediol degradation	*glpD*	Glycerol-3-phosphate dehydrogenase (aerobic)
pocR	Positive regulator of *cob* and *pdu*	*gltA*	Citrate synthase
traY	F plasmid DNA transfer functions	*icd*	Isocitrate dehydrogenase
		lctPRD	L-Lactate permease, regulator and L-Lactate dehydrogenase
		mdh	Malate dehydrogenase
		nuoA-N	NADH:quinone oxidoreductase
		pdhR-aceEf-lpd	Pyruvate dehydrogenase complex and regulator
		sdhCDAB	Succinate dehydrogenase
		sodA	Mn-Superoxide dismutase
		sucCD	Succinate thiokinase
		ND	D-amino acid dehydrogenase

All data from studies in *E.coli* and *S.typhimurium*.

2.7.3 Regulation of gene expression by NarXL and NarQP

Enteric bacteria carry out anaerobic respiration under anaerobic conditions with preferential use of terminal electron acceptor with a highest redox potential. A global regulatory system NarXL and NarQP is used to ensure highest yield of energy produced.

Reduction of chlorate into toxic compound chlorite is catalyzed by nitrate reductase. NarL was identified in an *E. coli* mutant resistant to chlorate with a basal nitrate activity (Stewart, 1988). *narL* gene can encode a specific nitrate-responsive positive regulatory element. NarL positive regulates nitrate reductase in the presence of nitrate under anaerobic conditions . *narX* gene was a closely linked gene to *narL*. Deduced amino acid sequences predicted that NarL is a response regulator and NarX is the cognate sensor of two-component system (Darwin and Stewart, 1996). However, mutation of NarX failed to abolish the effect of NarL-mediate gene regulation. In a *narX* mutant, another mutation was subsequently isolated that nitrate regulation was totally eliminated. The mutation was designated as *narQ* (Darwin and Stewart, 1996). In a *narL* null mutant, expression of formate dependent nitrite reductase is still nitrate-inducible, suggesting that another response regulator is present and subsequently *narP* was identified as the regulator (Darwin and Stewart, 1996). Therefore, there are 2 two-component regulatory system for respiratory control in the *E.coli.* A clear difference of functions of NarX and NarQ was identified when investigating operons regulated by nitrate or nitrite mediated by Nar system. In a *narX* mutant, the weak nitrite induction of *narG* and *fdnG* operon expression is increased and nitrite is as efficient as nitrate. This effect cannot be observed in *narQ*

mutant. It was proposed that in the presence of nitrite, the NarX protein negatively regulates NarL protein activity. The negative influence of the NarX protein is essential for normal nitrite regulation (Reviewed by Darwin and Stewart, 1996). Candidates of Nar modulon are listed in Table 2.5. The candidates can be classified into two classes: those regulated by NarL alone and those regulated by NarL and NarP. Most of them are regulated by Fnr also. A consensus sequence TACYNMT (where Y = C or T and M = A or C) was proposed to be important for NarL binding. The effect of NarL on the expression of some of these operons is differential, especially on *narG*, *fdnG* and *frdA*. The effect is largest on *narG* while least on *frdA*. This differential effect was proposed to be due to different affinities of NarL binding to the control regions (Reviewed by Darwin and Stewart, 1996).

Table 2.5 The Nar modulon in *E. coli* (modified from Darwin and Stewart, 1996).

NarL[a]	NarP	Gene	Enzyme
+	0	*nraGHJI*	Nitrate reductase
+	0	*narK*	Nitrite extrusion
+	+	*fdnGHI*	Formate dehydrogenase-N
+	+	*nirBDC*	NADH-nitrite reductase
+	+	*nuoA-N*	NADH-dehydrogenase
+	+	*narXL*	Nitrate/nitrite regulation
+	?	*modABCD*	Molybdate uptake
±[b]	+	*nrfABCDEFG*	Formate-nitrite reductase
-	0	*frdABCD*	Fumarate reductase
-	+	*nap-ccm*	Periplasmic nitrate reductase
-	-	*pfl*	pyruvate formate lyase
-	?	*adhE*	Alcohol dehydrogenase
-	?	*dmsABC*	DMSO reductase

a: +, positive regulation; -,negative regulation; 0, no effect; ?, not determined
b: NarL repress *nrfA* in the presence of nitrate and activate *nrfA* in the presence of nitrite.

2.7.4 Other aspects in anaerobic gene regulation

Most operons involved in anaerobic metabolism are not regulated by a single global regulator. These operons are subjected to dual or multifactorial control and belong to more than one modulon. For instance, Fnr modulon can overlap with Arc modulon, Nar modulon, Crp (carbon metabolism) modulon, Fur (iron uptake) modulon and SoxS (redox stress) modulon (Guest, 1995). Hence, the expression of a single operon is subjected to various environmental stimuli and is adjusted to suit a wide range of conditions. Moreover, anaerobiosis can influence DNA supercoiling and it also affect gene expression in anaerobiosis (Lin and Iuchi, 1991).

2.7.5 Relationship between NrdD and anaerobic metabolism

Most of the enzymes involved in the central metabolic pathways are under control of global regulators Fnr, ArcAB, NarXL and NarQP as discussed above. However, the regulation of expression of proteins important for biosynthetic or regulatory functions under anaerobic conditions are less characterized. Although anaerobic ribonucleotide reductase, NrdD, is not an enzyme involved in the central metabolic pathway, it plays an important role in anaerobic metabolism of enteric bacteria since it provides the dNTPs pool for DNA synthesis to the bacterial cell under anaerobic conditions. A putative Fnr binding site was proposed to be located at the promoter region of the *E. coli nrdD* gene (Sun *et al.*, 1993). It is an interesting problem whether *nrdD* would express differentially under anaerobic condtions. The study of this problem opens up a new window to the understanding of the regulatory mechanism of anaerobic metabolism of enteric bacteria.

2.8 Objectives

The gene encoding the enzyme responsible for anaerobic ribonucleotide reduction was cloned and sequenced in *E. coli* (Sun *et al.*, 1993). Moreover, *nrdD* was also found to present in two other living organisms, bacteria *Haemophilus influenzae* and archaeon *Methanococcus jannaschii*, whose genomes were being recently completely sequenced (Fleischmann *et al.*, 1995; Bult *et al.*, 1996),. However, in *S. typhimurium*, which is phylogenetically very close to *E. coli*, there has been no report of this gene although this bacterium can also live in anaerobic environment. The objectives of this study are:

1. To clone and sequence the genes encoding anaerobic ribonucleotide reductase, *nrdD* and its activase, *nrdG,* from *S. typhimurium*.
2. To study the regulation of *nrdD* in response to oxygen and different genetic backgrounds.
3. To characterize the function of NrdD by investigating the growth pattern of a mutant defective in *nrdD*.

Cloning and sequencing of *nrdDG* provide fundamental information to a few aspects of studies such as the structural properties of the proteins.

As most of the current studies in aerobic/anaerobic regulated genes are focused on central metabolic pathways, study of expression of *nrdD* would open up a new window to this interesting problem. This study targets on the unknown regulatory mechanism of *nrdD* gene expression with the ambition of sorting out the puzzle of the regulatory network of anaerobiosis.

Chapter 3

Molecular cloning and sequencing of *nrdDG* operon from *Salmonella typhimurium*

3.1 Introduction

Enteric bacteria such as *Escherichia coli* and *S. typhimurium* can live in both aerobic and anaerobic environments. They possess two different kinds of ribonucleotide reductase for deoxyribonucleotides biosynthesis. The anaerobic ribonucleotide reductase (NrdD) was identified in anaerobically grown *E. coli* (Fontecave *et al.*, 1989). This enzyme needs to be activated by a small protein NrdG (Sun *et al.*,1995). However, the homologues of these two enzymes have not been identified in *S. typhimurium* so far. Cloning and sequencing of the genes encoding anaerobic ribonucleotide reductase and its activase of *S. typhimurium* can help to understand the anaerobic biosynthesis of deoxyribonucleotides in this bacterium. Although the 5' untranslated region of the *nrdD* gene of *S. typhimurium* had been sequenced (Wong, 1990), the coding region and the downstream sequence of *nrdD* as well as *nrdG* have not yet been determined.

Four lambda clones containing the *nrdDG* genes were obtained from Dr. K.K. Wong at Pacific Northwest National Laboratory, Richland, WA, USA. Long distance polymerase chain reaction (LD-PCR) was first used to amplify the gene by one gene specific primer and one vector specific primer from each of the four clones. The LD-PCR product was digested with restriction enzymes and subcloned for sequencing. Both strands of the template were sequenced using vector specific primers and gene specific primers. The LD-PCR product was also sequenced directly.

3.2 Materials and Methods

3.2.1 Bacterial strains and bacteriophages.

Escherichia coli strain LE392 (F^- *supF supE hsdR galK trpR metB lacY tonA*) was used as the host for propagation of lambda phages. Epicurian Coli® XL-1 MRF' Kan supercompetent cells (Stratagene) were used for transformation and maintenance of plasmid vector and recombinant plasmids. Lambda DASH II (Stratagene) clones, 5E6, 1098, 882 and 10F11, which carry *nrdDG* structural genes of *Salmonella typhimurium* LT2, were used for subcloning.

3.2.2 Culture media.

LB (Luria-Bertani) medium contained 10 g Bacto-tryptone, 5 g Bacto-yeast extract and 10 g NaCl per liter. An additional 15 g agar per liter was added for solid plates. Top agar contained 7.5 g agar per liter. SOC medium contained 2% Bacto-tryptone, 0.5% Bacto-yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose.

3.2.3 Preparation of lambda lysate and phage DNA.

3.2.3.1 Plating out of lambda phage and preparation of plate lysate.

Each of the filter paper disks soaked with lambda phages, obtained from Dr. K. K. Wong, was immersed in 1 ml SM buffer (0.1 M NaCl, 8 mM $MgSO_4$, 50 mM Tris-HCl [pH 7.5], 0.01% gelatin). Ten µl of this solution was mixed with 200 µl overnight culture of LE392 in LB supplemented with 0.2% maltose and incubated at 37°C for 15

minutes. Three ml melted top agar was added to the mixture and poured onto a freshly prepared LB plate. The plate was incubated overnight at 37°C.

Plate lysate was prepared by adding 3 ml SM buffer to the plate and kept at 4°C for overnight. The suspension was collected. Cell debris was removed by centrifuging at 2000 rpm for 10 minutes. The supernatant was retained as the plate lysate.

3.2.3.2 Preparation of lambda lysate stock.

Each of the single isolated plaques was picked and suspended in 0.3 ml adsorption buffer (10 mM $MgCl_2$ and 10 mM $CaCl_2$)and mixed with 0.2 ml exponentially grown LE392 in LB medium with 0.4% maltose. The mixture was incubated at 37 °C for 10 minutes and 10 ml LB medium supplemented with 10 mM $MgCl_2$ and 0.1% glucose was added. The mixture was incubated at 37°C with shaking overnight. Bacterial debris was removed by centrifuging at 2000 rpm for 10 minutes. The supernatant was retained as the lysate stock.

3.2.3.3 Preparation of lambda phage DNA.

Lambda DNA was prepared as described (Malik *et al.*, 1990) with some modifications. Three ml plate lysate was added to 100 ml

exponentially grown LE392 in LB medium supplemented with 10 mM $MgCl_2$ and 0.2% maltose. The culture was shaken for about 3 hours at 37°C until lysis. After addition of 0.5 ml chloroform, the culture was shaken for 15 minutes. The lysed culture was added with 4 g NaCl, 100 µl RNase A (10 mg/ml), and 100 µl DNase I (10 mg/ml) were added to the culture and incubated at 37°C for 1 hour. The mixture was centrifuged at 3000 rpm for 10 minutes. The supernatant was added with 10 g PEG8000 to precipitate the phage particles. The mixture was placed in ice-water bath for 1 hour and then centrifuged at 3000 rpm for 20 minutes. The supernatant was discarded. The phage pellet was suspended in 4 ml SM buffer with the addition of 40 µl RNase A (10 mg/ml) and 40 µl DNase I (10 mg/ml). The suspension was incubated at 37°C for 30 minutes. The phage was lysed by incubating with 80 µl SDS (10% w/v) and 40 µl protease K (10 mg/ml) at 37°C for 45 minutes. The suspension was then extracted with phenol : chloroform : isoamyl alcohol (25:24:1) twice and once with chloroform : isoamyl alcohol (24:1). Phage DNA was precipitated from the aqueous phase by adding 1/10 volume of 3M sodium acetate (pH 5.2) together with 2 volumes of 100% ethanol at -20°C. DNA pellet was washed with 70% ethanol and dried under vacuum. The DNA pellet was dissolved in 0.5 ml ultra-pure water. The quality of the phage DNA was checked by 1% agarose gel electrophoresis in 1X TBE buffer. The quantity of the phage DNA was measured by UV

spectrophotometry at 260 and 280nm using GeneQuant machine (Pharmacia).

3.2.4 Long distance polymerase chain reaction (LD-PCR) of *nrdDG* gene fragment.

Lambda phage DNA prepared above was used as the template in LD-PCR. PCR was performed in 50 µl of 1X *Taq* Extender™ reaction buffer (20 mM Tris-HCl [pH 8.8], 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton® X-100 and 0.1 mg/ml nuclease-free bovine serum albumin), 0.8 mM each of dNTPs (Pharmacia), 0.2 µM primer nrdDup2 (AAATGCACACCGACCCAATA), 0.2 µM of either primer T3 (AATTAACCCTCACTAAAGGG) or T7 (GTAATACGACTCACTATAGGGC), 0.1 µg template phage DNA, 5 units *Taq* DNA polymerase (Promega) and 5 units *Taq* Extender™ PCR Additive (Stratagene). Touchdown PCR was adopted for the amplification with a programmable thermal cycler PTC-100 (MJ research). The PCR profile was composed of 1 cycle of denaturation at 94°C for 1 minute, followed by 19 touchdown cycles of denaturation at 94°C for 30 seconds, primer annealing at 68 °C for 1 minute with 1°C decrease per cycle and elongation at 72°C for 10 minutes. The touchdown cycles were followed by 16 cycles of denaturation at 94°C for 30 seconds, primer annealing at 50°C for 1 minute and elongation at 72°C for 10 minutes. The PCR profile was finished by a final step of 72°C for 15 minutes was included. The PCR mixture was soaked at 15°C. 5 µl of PCR product was resolved on 1% agarose gel electrophoresis in 1X TBE buffer. The LD-PCR product was used for direct

sequencing or digested with restriction enzymes and subcloned into the plasmid vector.

3.2.5 Restriction enzyme digestion of LD-PCR products and subcloning of restriction fragments.

Restriction enzyme digestion was performed with 1X One-Phor-All Buffer PLUS (10 mM Tris-acetate [pH 7.5], 10 mM Magnesium acetate and 50 mM potassium acetate), 10 µl LD-PCR product and 15 units of restriction enzyme *Hpa* I (5'-GTT↓AAC-3', Pharmacia) in a 20 µl reaction volume. The mixture was incubated at 37°C for 1 hour and then the enzyme was inactivated at 85°C for 30 minutes. After heating , the mixture was cooled at room temperature. The restriction fragments were resolved on 1% agarose gel electrophoresis in 1X TBE buffer.

The restriction fragments were subcloned into the pCR-script™ Amp SK (+) cloning vector by using the kit from Stratagene. The subcloning reaction was performed by mixing 10 ng of pCR-script™ Amp SK (+) cloning vector, 1 µl pCR-Script 10X reaction buffer, 0.5 µl 10 mM ATP, 2 µl restriction fragments, 1 µl *Srf* I restriction enzyme (5 U/ µl) and 1 µl T4 DNA ligase (4 U/ µl) in a 10 µl reaction volume. The reaction was incubated at room temperature for 1 hour and inactivated at 65°C for 10 minutes.

The ligation mixture was transformed into Epicurian Coli® XL-1 MRF' Kan supercompetent cells (Stratagene). Forty µl frozen competent cells was thawed on ice and aliquoted into prechilled Falcon polypropylene tubes. β-mercaptoethanol was

added to each tube of the cell to a final concentration of 25 mM. The mixture was incubated on ice for 10 minutes and swirled every 2 minutes. Two µl of the ligation mixture was added to the tube and incubated on ice for 30 minutes. The mixture was heat pulsed at 42°C for 45 seconds and returned to ice for 2 minutes. SOC (0.45 ml, pre-equilibrated at 42°C) was added to the tube. The mixture was incubated at 37°C for 1 hour with shaking at 225-250 rpm. Portions of 100 µl and 200 µl mixture were spread onto LB plates containing 100 µg/ml ampicillin, 40 mg/ml X-gal and 40 mg/ml IPTG. The plates were incubated at 37°C for overnight. White colonies were identified as cells carrying recombinant plasmids.

3.2.6 Confirmation of recombinants by colony-PCR

White colonies were picked and immersed into PCR reaction mix which contained 1X Reaction buffer (50 mM Tris-HCl [pH 9.0] and 20 mM ammonium sulphate, Epicentre Technologies), 2.5 mM $MgCl_2$, 0.8 mM each dNTPs, 0.4 µM primer T3 (AATTAACCCTCACTAAAGGG) , 0.4 µM primer T7 (GTAATACGACTCACTATAGGGC) and 0.2 units of *Tfl* DNA polymerase (Epicentre Technologies) in a 20 µl reaction volume. The PCR reaction was performed on a programmable thermal cycler PTC-100 (MJ research) with a PCR profile which was composed of 1 cycle of 95°C for 5 minutes, 30 cycles of 1 minute denaturation at

94°C, 1 minute primer annealing at 55°C and 1 minute elongation at 72°C. PCR products were resolved by 1% agarose gel electrophoresis in 1X TBE buffer.

3.2.7 Preparation of plasmid DNA by alkaline lysis using Wizard™ *Plus* Miniprep DNA Purification System (Promega)

Alkaline lysis method was used to extract plasmid DNA from bacterial cells by using Wizard™ *Plus* Miniprep DNA Purification System from Promega. Three ml overnight culture of cells carrying recombinant plasmids in LB with 100 µg/ml ampicillin was centrifuged at 13000 rpm for 2 minutes, the supernatant was removed and the bacterial pellet was suspended in 200 µl Cell Resuspension Solution (50 mM Tris-HCl [pH 7.5], 10 mM EDTA and 100 µg/ml RNase A). The cell suspension was lysed with 200 µl Cell Lysis Solution (0.2 M NaOH and 1% SDS). The mixture was shaken gently until clear. To the cleared lysate, 200 µl Neutralization Solution (1.32M potassium acetate) was added. The mixture was centrifuged at 13000 rpm for 5 minutes. At the same time, one Wizard™ Miniprep Column (Promega) was attached to a 3-ml syringe barrel, the tip of the Minicolumn/Syringe barrel assembly was inserted into a Promega Vac-Man™ Laboratory Vacuum Manifold and the stopcock of the manifold was closed. One ml Wizard™ *Plus* Miniprep DNA Purification Resin (Promega) was pippetted into the syringe barrel. The cleared lysate from each miniprep after centrifugation was transferred into each barrel of the assembly containing the resin. The stopcock of each column was opened and vacuum was applied, after the resin had been completely passed through the column, the

vacuum was stopped. Two ml of Column Wash Solution (80 mM potassium acetate, 8.3 mM Tris-HCl [pH 7.5], 40 μM EDTA and 55% ethanol) was added to the barrel and vacuum was applied. After the solution had been pulled through the column, the resin was dried for another 30 seconds. The Minicolumn was detached and transferred into a microcentrifuge tube and centrifuged at 13000 rpm for 2 minutes to remove residual ethanol. Then, 50 μl water was added to the Minicolumn and soaked for 1 minute. Plasmid DNA was eluted from the column by centrifuging at 13000 rpm for 20 seconds. Plasmid DNA was resolve by 1% agarose gel electrophoresis in 1X TBE buffer. The quantity of the plasmid DNA was measured by UV spectrophotometry at 260 and 280nm using GeneQuant machine (Pharmacia).

3.2.8 DNA cycle sequencing by using dye-labeled dideoxy chain terminator and data collection.

The ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase, FS (Perkin Elmer) was used to determine the nucleotide sequence. Either plasmid DNA or the LD-PCR product was used as template for sequencing reaction. The LD-PCR product was first purified by Centricon-100 column (Amicon). Centricon-100 column was assembled according to manufacturer's recommendations. The cycle sequencing was performed in a 20 μl reaction which contained 250-500 ng of double-stranded plasmid DNA or 100-200 ng purified LD-PCR product, 8 μl Terminator Ready Reaction Mix (A-Dye Terminator, C-Dye Terminator, G-Dye Terminator, T-Dye Terminator, dITP, dATP, dCTP,

dTTP, Tris-HCl [pH 9.0], $MgCl_2$, thermostable pyrophosphatase, and AmpliTaq DNA Polymerase, FS) and 3.2 pmoles of primer (Table 3.1). The cycle sequencing was performed on a GeneAmp PCR System 2400 (Perkin Elmer), the thermal cycling profile was composed of 25 cycles of 10 seconds denaturation at 96°C, 5 seconds primer annealing at 50°C and 4 minutes elongation at 60°C. Then, the cycle sequencing reaction product was purified by ethanol precipitation. Either one-tenth of 3M sodium acetate (pH 5.2) or half volume of 7.5M ammonium acetate was added to the cycle sequencing reaction, then, 2 volumes of 95% ethanol was added, the mixture was mixed and placed on ice for 15-60 minutes, then, the mixture was centrifuged at 13000 rpm for 15-30 minutes. The supernatant was removed and the pellet was washed with 250 µl 50% ethanol. The pellet was then air dried and suspended in 25 µl template Suppression Reagent (Perkin Elmer). The suspension was vortexed, centrifuged and heated to denatured the DNA at 95°C for 2. The sample was then vortexed, centrifuged, transferred to a 0.5 ml Genetic Analyzer Sample tube (Perkin Elmer), covered with Genetic Analyzer septum (Perkin Elmer) and put on the autosampler tray. Capillary electrophoresis was performed by ABI PRISM B310 to resolve the cycle sequencing products in 1X Genetic Analysis Buffer (Perkin Elmer) and at default settings (Injection at 2.4 kV for 30 seconds, running at 7.5 kV at 42 °C for 140 minutes). The raw data of the sequencing reaction generated by the sequencer was collected by ABI PRISM B310 Genetic Analyzer Data Collection software (Perkin Elmer). Raw data was compiled and analyzed by ABI PRISM B310 Genetic Analyzer Sequencing Analysis software (Perkin Elmer). Each processed sequence data was stored as one sequence text file and one sample file

which contained the raw data, analyzed data (chromatograph and nucleotide sequence), electrophorogram, and the file information.

3.2.9 Computer softwares for analyzing and manipulating DNA sequences.

SeqEd version 1.0.3 designed by Applied Biosystems (ABI) was employed to view, edit, align, and compare sequence data generated by ABI PRISM B310 Genetic Analyzer. The sample file was edited directly using this software. Each sample sequenced by the same primer was imported into same layout window, sample corresponding to the complementary strand was first translated into a reverse complement sequence and imported. These sequences were aligned (comparative mode) and a consensus sequence was obtained. This consensus sequence was exported as the final reference sequence. Consensus sequences from different sequencing reactions can be imported in the same layout window and aligned with each other (contig mode).

GeneJockey (Biosoft) was used to manipulate nucleotide sequences, translate nucleotide sequences into amino acid sequences, search for open reading frames, align nucleotide sequences and perform restriction digestion analysis.

Oligo 4.05 (National Biosciences, Inc.) was used to search for PCR or sequencing primers. Primers were chosen according to its specificity (*T*m ranged from 50 °C to 70 °C), absence of hairpin structure or dimer formation. Optimal PCR annealing temperatures were also determined by this program if possible.

TABLE 3.1 Primers used in DNA sequencing.

Primer name	Primer sequence
nrdDup2	AAA TGC ACA CCG ACC CAA TA
nrdDlp1	GCC ATC TCG TTT CAT CAC AT
nrdDlp2	GTC ATC GAC TCC CGC TGC TT
nrdDcdup1	TCG CAG AAG TCG TTA GCA G
nrdDcdup1rev	CTG CTA ACG ACT TCT GCG A
nrdDcdlp1	TGA ACA TCG GGA AGA ACG G
nrdDcdlp1rev	CCG TTC TTC CCG ATG TTC A
nrdDcdup2	GAT TAG GTG GTC AAA GTC A
nrdDcdup2rev	TGA CTT TGA CCA CCT AAT C
nrdDcdlp2	GTC GTG GTT GCC GCA TTT C
nrdDcdlp2rev	GAA ATG CGG CAA CCA CGA C
nrdGup1	TTT CTG GAC ACC CGC TAT GC
nrdGup1rev	GCA TAG CGG GTG TCC AGA AA
nrdGup2	AAC TCA ACG CCG CAC AGA TG
nrdGup2rev	CAT CTG TGC GGC GTT GAG TT
nrdD728up	ACC GCA AAA CCG CCG ATG AG
nrdD728lp	CTC ATC GGC GGT TTT GCG GT
nrdD1522up	TAC GAC AGC GAG CAA CTT C
nrdD1522lp	GAA GTT GCT CGC TGT CGT A
T3	AAT TAA CCC TCA CTA AAG GG
KS	TCG AGG TCG ACG GTA TC
M13	GTA AAA CGA CGG CCA GT
M13 reverse	GGA AAC AGC TAT GAC CAT G

(Primers were synthesized from either Integrated DNA Technologies, Inc. or GENSET Corp.)

3.3 Results

3.3.1 Preparation of lambda DNA.

5E6, 1098, 882 and 10F11 were four lambda DASH II clones which contained the *nrdDG* gene of *S. typhimurium*. They were isolated by Dr. K. K. Wong at Pacific Northwest National Laboratory, Richland, WA, USA. Lambda DNA was isolated from liquid lysate of these clones and resolved by agarose gel electrophoresis as (Fig. 3.1). The sizes of the DNA isolated from each clone were all greater than 23 kb. The concentration of DNA was about 3-5 μg/μl. The OD260 to OD280 ratio was 1.86.

3.3.2 Long distance PCR amplification of *nrdDG* from lambda DNA.

Since the orientation of *nrdDG* of each clone was not known, both vector specific primers, T3 and T7, were chosen to amplify the genes. Either T3 or T7 primers can amplify the *nrdDG* gene with a gene specific primer, nrdDup2, from each clone. The LD-PCR products resolved by agarose gel electrophoresis were shown in Fig. 3.2. From 5E6, a 2.3 kb PCR product was amplified using T7 and nrdDup2. Amplified with T3 and nrdDup2, LD-PCR products from 10F11 was about 10 kb; from 1098, about 9kb; and from 889, also about 9 kb. Non-specific bands were obtained in LD-PCR of 5E6, while the others gave only one specific amplicon.

Fig. 3.1 DNA prepared from different lambda clones resolved by 1% agarose gel electrophoresis. Lane M: lambda *Hind* III cut DNA, Lane1: clone 5E6, lane2: clone 10F11, lane3: clone 1098, lane4: clone 882

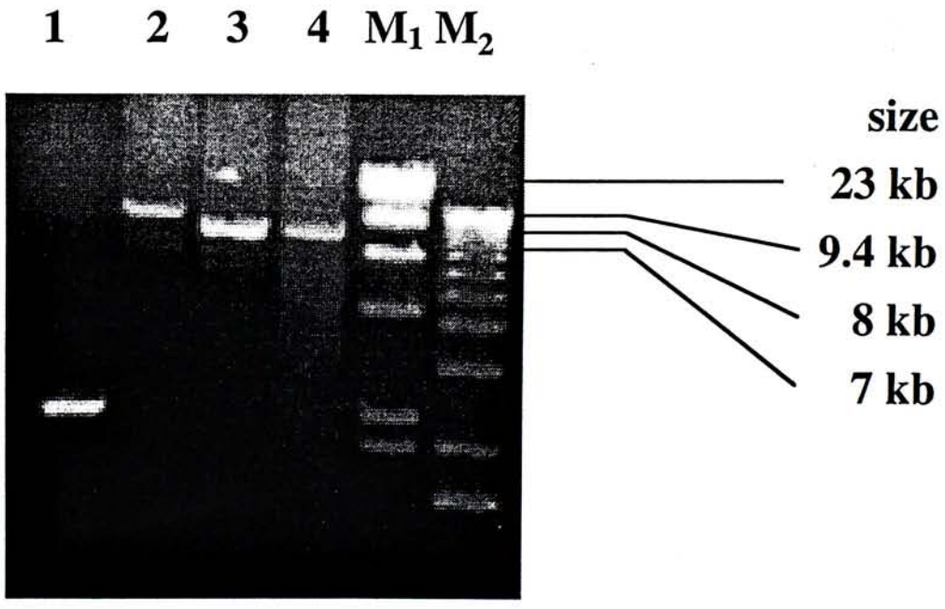


Fig. 3.2 Long distance PCR products amplified with primers nrdDup2 and T3/T7 on different lambda clones resolved by 1% agarose gel electrophoresis. Lane1: clone 5E6, lane2: clone 10F11, lane3: clone1098, lane4: clone 882 , lane M_1:lambda *Hind* III cut DNA, lane M_2: 1 kb ladder (Gibco BRL)

3.3.3 Restriction digestion of LD-PCR products

The LD-PCR products from 10F11, 1098 and 882 were digested with *Hpa* I. It is a six base blunt-end restriction enzyme. The 10F11 and 1098 LD-PCR products were each digested into three restriction fragments of 6 kb, 1.5 kb and 1 kb. An additional 0.5 kb fragment was obtained from 882 LD-PCR products (Fig. 3.3).

3.3.4 Subcloning of LD-PCR restriction fragments.

The restriction fragments were then ligated with *Srf* I digested pCR-script vector DNA and transformed into XL-1 Blue cells. Number of transformants were shown in Table3.2. Since in each transformation, about 100 ng DNA was used, the transformation efficiency was about 10^4 to 10^5/μg DNA.

A total of 96 colonies were picked and screened for recombinant plasmids, fifteen white colonies and one blue colonies were picked from each plate. Out of these 96 colonies, 20 of them were shown to carry recombinant plasmids. PCR products of 1.6 kb , 1.1 kb or 0.6 kb were amplified from one of these clones (Fig. 3.4). The increase of 0.1 kb over the insert was due to the flanking sequence of the multiple cloning site in the vector. Clones carrying the 6 kb restriction fragment cannot be identifed. It may probably due to low cloning efficiency for large DNA fragment. Morever, PCR screening cannot detect insert greater than 2 kb in size.

LD-PCR product from	vol. plated (µl)	total no. of transformants	no. of blue colonies	no. of white colonies
10F11	100	500	200	300
	200	800	300	500
1098	100	300	100	200
	200	500	200	300
882	100	200	100	100
	200	400	200	200

Table. 3.2 Number of Transformants on subcloning of different *Hpa* I digested LD-PCR products. (Figures are rounded-up to nearest hundreds)

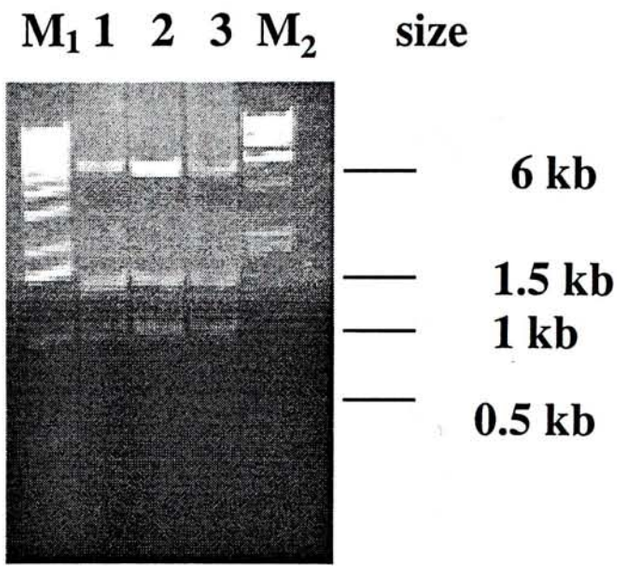


Fig.3.3 Restriction fragments of long distance PCR products cut by *Hpa* I resolved by agarose gel electrophoresis. Lane M_1: 1 kb ladder (Gibco BRL). Lane1: DNA from10F11. Lane2: DNA from 1098. Lane3: DNA from 882. Lane M_2: lambda *Hin*d III cut. Note that the 0.5 kb fragment cannot be clearly seen in this photo.

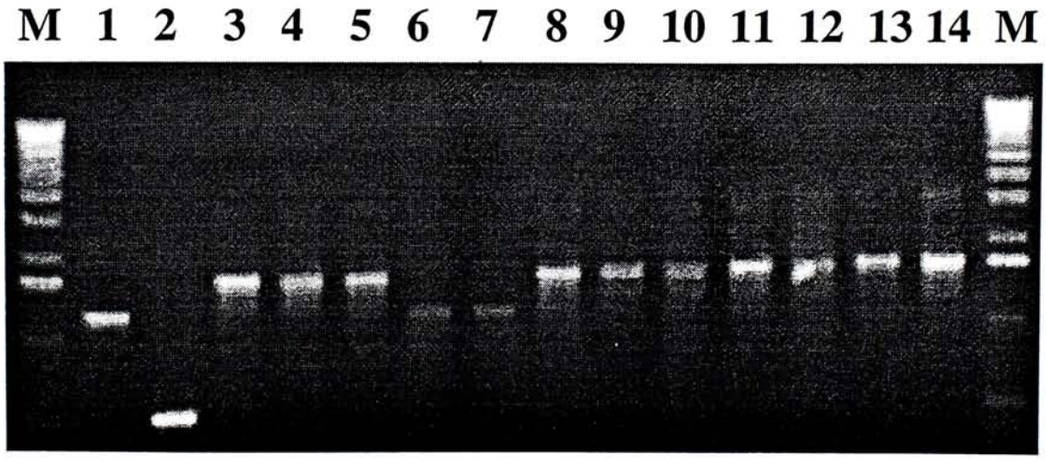


Fig. 3.4 Positive clones were screened by colony PCR. Clones carrying recombinant plasmids would give PCR products of correct sizes. Lanes1,6 and 7: Clones carrying an 1 kb insert. Lanes 2: clone carrying a 0.5 kb insert. Lanes3-5, 8-14: Clones carrying 1.5 kb insert. (size included 0.1 kb of flanking sequence.) Lane M: 1 kb ladder (Gibco BRL)

3.3.5 Miniprep of plasmid DNA from recombinants and verification of *nrdDG* identities.

Eight recombinants were picked and chosen for miniprep of plasmid DNA. Four of them carried inserts of 1.5 kb (clones 8, 9, 10 and 14), three of them carried inserts of 1 kb (clones 4, 11 and 13) and one of them carried an insert of 0.5 kb (clone 6). The concentrations of the plasmids ranged from 0.05 μg/μl to 0.2 μg/μl. Fig. 3.5 showed the plasmid DNA resolved by agarose gel electrophoresis. Clones 8, 11 and 6 were partially sequenced using vector specific primers M13 and M13 reverse to verify the presence of *nrdDG*. Where the partial nucleotide sequences of these three clones were searched against the GenBank database of the BLAST WWW Server by using National Centre for Biotechnology Information (NCBI) using BLASTN as well as BLASTX (Altschul *et al.*, 1990), only clones 8 and 11 showed homology to *E. coli nrdD* but not clone 6 (It matched riboflavin synthase alpha chain of *Bacillus subtilus* but at a low similarity [20%]). Clone 11 can matched the 5' untanslated and coding region of *E. coli nrdD* of (Sun *et al.*, 1993) and Clone 8 matched the 3' coding and untranslated regions. Therefore, clones 11 and 8 appeared to contain the *nrdD* of *S. typhimurium* and designated as pNWL11 and pNWL8 respectively. Both clones were used for nucleotide sequence determination. However, *nrdG* was not present in these clones and another approach was used to sequence the gene as described below.

M 1 2 3 4 5

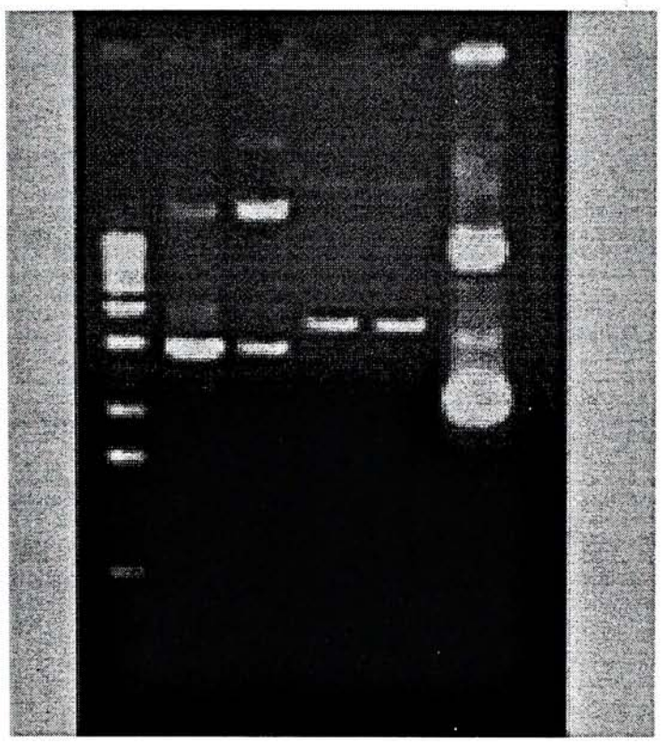


Fig.3.5 Miniprep of plasmids from positive clones resolved by 1% agarose gel electrophoresis. Lane M: 1 kb ladder (Gibco BRL). Lane1-2: Plasmids carrying an 1 lb insert. Lane 3-4: Plasmids carrying an 1.5 kb insert. Lane5: Plasmid carrying a 0.5 kb insert

3.3.6 Nucleotide sequence of *nrdDG*.

Although pNWL11 and pNWL8 contained the *nrdD* of *S. typhimurium*, a single run of sequencing reaction (about 300-400 bp) could not reveal the complete nucleotide sequences of the genes. Moreover, a single primer can only determine one strand of DNA sequence. Different primers were thus designed to determine the complete nucleotide sequence of *nrdD* of *S. typhimurium* in both DNA strands. The position of primers used in sequencing are shown in fig.3.6. Another concern was that the two clones did not cover the *nrdG* of *S. typhimurium*. To solve the problem, the LD-PCR product was used as the template for direct sequencing. Since in *E. coli*, the length of *nrdDG* operon is about 3 kb, if *S. typhimurium* has a homologue with similar length, LD-PCR products longer than 9 kb (10F11, 1098 or 882) should be able to cover the *nrdG* gene.

A total of 2994 bp were sequenced, two putative open reading frames (ORF) were identified by GeneJockey software. The complete nucleotide sequence and the amino acid sequence translated are shown in Fig. 3.6. The first ORF was 2136 bp long starting from 187 bp to 2322 bp, which would encode a protein with 712 amino acid residues. The start codon of this ORF was ATG (Met) while the termination codon was TAA (Orche). The second ORF was 462 bp long starting from 2446 bp to 2907 bp, which would encode a protein with 154 amino acid residues. The start codon of the second ORF was also ATG (Met) but the termination codon was TGA (Umber). The intervening sequence between these two ORFs contained 123 bp. There was no ORF identified downstream of the second ORF. The predicted molecular weights of the protein encoded by the first ORF and the second ORF were

80 kDa and 17.4 kDa. These two ORFs were searched for homology in the NCBI GenBank database using BLASTX (Altschul *et al.*, 1990). These two ORFs matched several protein coding regions of different organisms with different overall amino acid identities (Table 3.3a and b).

Table 3.3a Homology search results of the first ORF using BLASTX.

Organism	Enzyme matched	Identities
E. coli	anaerobic ribonucleoside-triphosphate reductase	93%
Haemophilus influenzae Rd	anaerobic ribonucleoside-triphosphate reductase	72%
Bacteriophage T4	anaerobic ribonucleoside-triphosphate reductase	51%
Methanococcus jannaschii	anaerobic ribonucleoside-triphosphate reductase	40%
Pyrococcus furiosus	ribonucleotide reductase	38%
Thermoplasma acidophilum	ribonucleotide reductase	30%

Table 3.3b Homology search results of the second ORF using BLASTX.

Organism	Enzyme matched	Identities
E. coli	anaerobic ribonucleoside-triphosphate reductase activating protein	92%
H. influenzae Rd	anaerobic ribonucleoside-triphosphate reductase activating protein	59%
Bacteriophage T4	anaerobic ribonucleoside-triphosphate reductase activating protein	48%
H. influenzae Rd	pyruvate formate-lyase 1 activating enzyme	38%
Clostridium pasteurianum	iron-dependent pyruvate formate-lyase activating enzyme	36%
E. coli	pyruvate formate-lyase 1 activating enzyme	36%

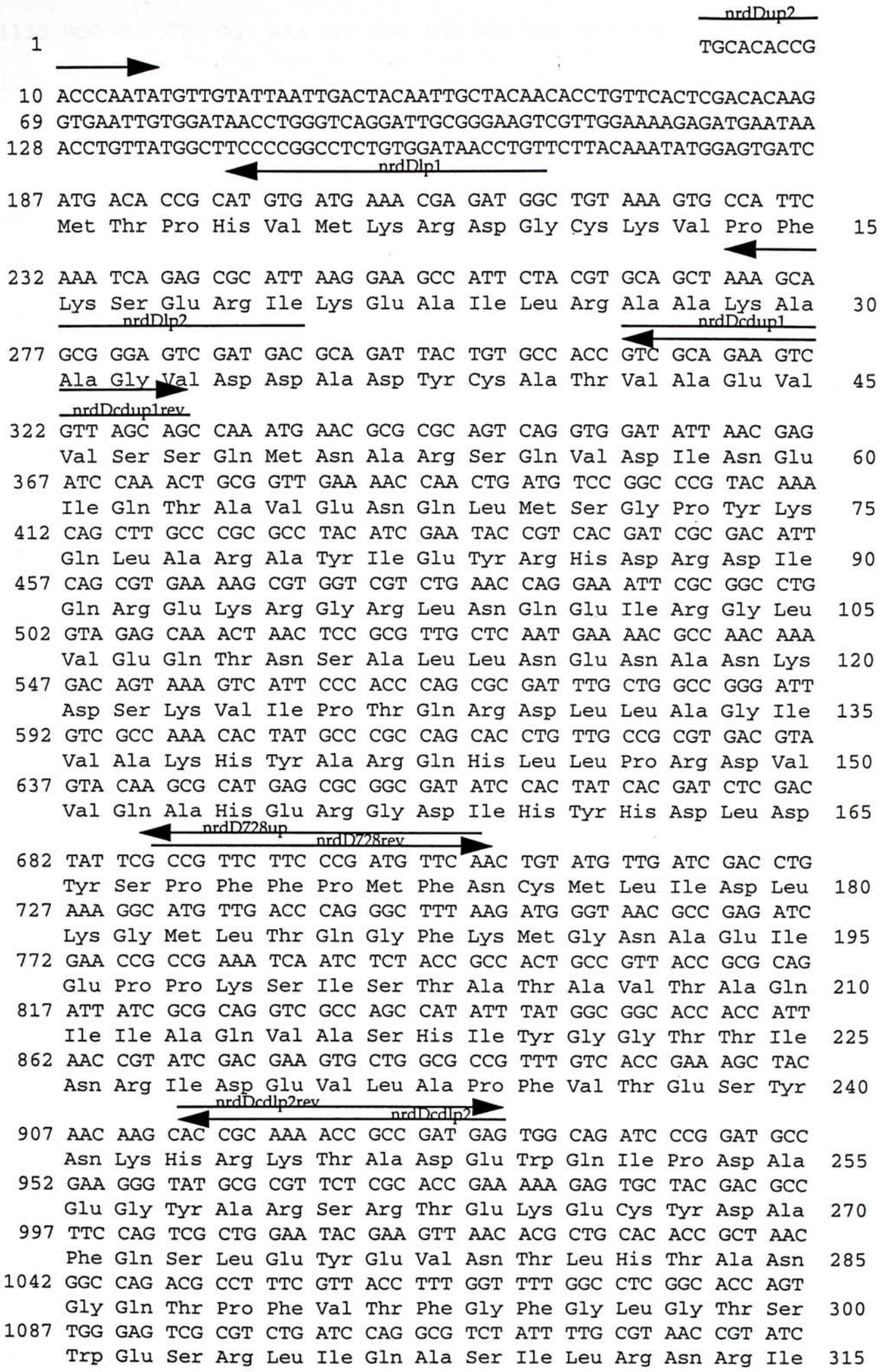

nrdDup2
1 TGCACACCG
10 ACCCAATATGTTGTATTAATTGACTACAATTGCTACAACACCTGTTCACTCGACACAAG
69 GTGAATTGTGGATAACCTGGGTCAGGATTGCGGGAAGTCGTTGGAAAAGAGATGAATAA
128 ACCTGTTATGGCTTCCCCGGCCTCTGTGGATAACCTGTTCTTACAAATATGGAGTGATC
nrdDlp1
187 ATG ACA CCG CAT GTG ATG AAA CGA GAT GGC TGT AAA GTG CCA TTC
Met Thr Pro His Val Met Lys Arg Asp Gly Cys Lys Val Pro Phe 15
232 AAA TCA GAG CGC ATT AAG GAA GCC ATT CTA CGT GCA GCT AAA GCA
Lys Ser Glu Arg Ile Lys Glu Ala Ile Leu Arg Ala Ala Lys Ala 30
nrdDlp2
nrdDcdup1
277 GCG GGA GTC GAT GAC GCA GAT TAC TGT GCC ACC GTC GCA GAA GTC
Ala Gly Val Asp Asp Ala Asp Tyr Cys Ala Thr Val Ala Glu Val 45
nrdDcdup1rev
322 GTT AGC AGC CAA ATG AAC GCG CGC AGT CAG GTG GAT ATT AAC GAG
Val Ser Ser Gln Met Asn Ala Arg Ser Gln Val Asp Ile Asn Glu 60
367 ATC CAA ACT GCG GTT GAA AAC CAA CTG ATG TCC GGC CCG TAC AAA
Ile Gln Thr Ala Val Glu Asn Gln Leu Met Ser Gly Pro Tyr Lys 75
412 CAG CTT GCC CGC GCC TAC ATC GAA TAC CGT CAC GAT CGC GAC ATT
Gln Leu Ala Arg Ala Tyr Ile Glu Tyr Arg His Asp Arg Asp Ile 90
457 CAG CGT GAA AAG CGT GGT CGT CTG AAC CAG GAA ATT CGC GGC CTG
Gln Arg Glu Lys Arg Gly Arg Leu Asn Gln Glu Ile Arg Gly Leu 105
502 GTA GAG CAA ACT AAC TCC GCG TTG CTC AAT GAA AAC GCC AAC AAA
Val Glu Gln Thr Asn Ser Ala Leu Leu Asn Glu Asn Ala Asn Lys 120
547 GAC AGT AAA GTC ATT CCC ACC CAG CGC GAT TTG CTG GCC GGG ATT
Asp Ser Lys Val Ile Pro Thr Gln Arg Asp Leu Leu Ala Gly Ile 135
592 GTC GCC AAA CAC TAT GCC CGC CAG CAC CTG TTG CCG CGT GAC GTA
Val Ala Lys His Tyr Ala Arg Gln His Leu Leu Pro Arg Asp Val 150
637 GTA CAA GCG CAT GAG CGC GGC GAT ATC CAC TAT CAC GAT CTC GAC
Val Gln Ala His Glu Arg Gly Asp Ile His Tyr His Asp Leu Asp 165
nrdD728up
nrdD728rev
682 TAT TCG CCG TTC TTC CCG ATG TTC AAC TGT ATG TTG ATC GAC CTG
Tyr Ser Pro Phe Phe Pro Met Phe Asn Cys Met Leu Ile Asp Leu 180
727 AAA GGC ATG TTG ACC CAG GGC TTT AAG ATG GGT AAC GCC GAG ATC
Lys Gly Met Leu Thr Gln Gly Phe Lys Met Gly Asn Ala Glu Ile 195
772 GAA CCG CCG AAA TCA ATC TCT ACC GCC ACT GCC GTT ACC GCG CAG
Glu Pro Pro Lys Ser Ile Ser Thr Ala Thr Ala Val Thr Ala Gln 210
817 ATT ATC GCG CAG GTC GCC AGC CAT ATT TAT GGC GGC ACC ACC ATT
Ile Ile Ala Gln Val Ala Ser His Ile Tyr Gly Gly Thr Thr Ile 225
862 AAC CGT ATC GAC GAA GTG CTG GCG CCG TTT GTC ACC GAA AGC TAC
Asn Arg Ile Asp Glu Val Leu Ala Pro Phe Val Thr Glu Ser Tyr 240
nrdDcdlp2rev
nrdDcdlp2
907 AAC AAG CAC CGC AAA ACC GCC GAT GAG TGG CAG ATC CCG GAT GCC
Asn Lys His Arg Lys Thr Ala Asp Glu Trp Gln Ile Pro Asp Ala 255
952 GAA GGG TAT GCG CGT TCT CGC ACC GAA AAA GAG TGC TAC GAC GCC
Glu Gly Tyr Ala Arg Ser Arg Thr Glu Lys Glu Cys Tyr Asp Ala 270
997 TTC CAG TCG CTG GAA TAC GAA GTT AAC ACG CTG CAC ACC GCT AAC
Phe Gln Ser Leu Glu Tyr Glu Val Asn Thr Leu His Thr Ala Asn 285
1042 GGC CAG ACG CCT TTC GTT ACC TTT GGT TTT GGC CTC GGC ACC AGT
Gly Gln Thr Pro Phe Val Thr Phe Gly Phe Gly Leu Gly Thr Ser 300
1087 TGG GAG TCG CGT CTG ATC CAG GCG TCT ATT TTG CGT AAC CGT ATC
Trp Glu Ser Arg Leu Ile Gln Ala Ser Ile Leu Arg Asn Arg Ile 315

Fig. 3.6

```
1132 GCC GGT CTG GGT AAA AAT CGC AAA ACC GCC GTG TTC CCG AAA CTG
     Ala Gly Leu Gly Lys Asn Arg Lys Thr Ala Val Phe Pro Lys Leu  330
1177 GTC TTC GCT ATC CGC GAT GGT TTG AAC CAC AAG TTT GGC GAT CCG
     Val Phe Ala Ile Arg Asp Gly Leu Asn His Lys Phe Gly Asp Pro  345
1222 AAC TAC GAC ATT AAA CAA CTG GCG CTG GAG TGC GCG AGC AAG CGC
     Asn Tyr Asp Ile Lys Gln Leu Ala Leu Glu Cys Ala Ser Lys Arg  360
                                   nrdcdup2
                                   ------->
                                   <-------
                                   nrdcdup2rev
1267 ATG TAT CCG GAC ATC CTC AAC TAC GAT CAG GTG GTC AAA GTC ACC
     Met Tyr Pro Asp Ile Leu Asn Tyr Asp Gln Val Val Lys Val Thr  375
1312 GGT TCG TTC AAA ACG CCA ATG GGC TGC CGC AGC TTC CTC GGC GTG
     Gly Ser Phe Lys Thr Pro Met Gly Cys Arg Ser Phe Leu Gly Val  390
1357 TGG GAG AAC GAA AAC GGC GAG CAG ATC CAC GAC GGG CGT AAC AAC
     Trp Glu Asn Glu Asn Gly Glu Gln Ile His Asp Gly Arg Asn Asn  405
1402 CTG GGC GTG ATT AGC CTT AAC CTG CCG CGC ATC GCG CTG GAA GCA
     Lys Gly Asp Glu Thr Ala Phe Trp Lys Leu Leu Asp Glu Arg Leu  435
1447 AAA GGC GAC GAA ACC GCA TTC TGG AAA CTG CTG GAT GAA CGT CTG
     Leu Gly Val Ile Ser Leu Asn Leu Pro Arg Ile Ala Leu Glu Ala  420
1492 GCG CTG GCG CGG AAG GCG CTG ATG ACC CGT ATC GCC CGC CTT GAA
     Ala Leu Ala Arg Lys Ala Leu Met Thr Arg Ile Ala Arg Leu Glu  450
1537 GGC GTG AAA GCC CGC GTC GCG CCT ATC CTG TAT ATG GAA GGC GCC
     Gly Val Lys Ala Arg Val Ala Pro Ile Leu Tyr Met Glu Gly Ala  465
1582 TGC GGC GTG CGG CTG AAA GCG GAC GAC GAC GTG TCT GAA ATC TTT
     Cys Gly Val Arg Leu Lys Ala Asp Asp Asp Val Ser Glu Ile Phe  480
1627 AAA AAT GGT CGT GCG TCC ATC TCT CTG GGC TAC ATT GGT ATC CAT
     Lys Asn Gly Arg Ala Ser Ile Ser Leu Gly Tyr Ile Gly Ile His  495
                                                  nrd1522up
                                                  <-------
1672 GAA ACC ATT AAC GCG CTG TTC GGC GGC GAA CAC CTG TAC GAC AGC
     Glu Thr Ile Asn Ala Leu Phe Gly Gly Glu His Leu Tyr Asp Ser  510
     ------->
     nrd1522lp
1717 GAG CAA CTT CGC GCT AAA GGC ATC GCG ATT GTG GAG CGT CTG CGC
     Glu Gln Leu Arg Ala Lys Gly Ile Ala Ile Val Glu Arg Leu Arg  525
1762 CAG GCC GTG GAT CAG TGG AAA GAC GAG ACC GGC TAC GGC TTT AGC
     Gln Ala Val Asp Gln Trp Lys Asp Glu Thr Gly Tyr Gly Phe Ser  540
1807 CTG TAC AGC ACG CCA AGT GAA AAC CTC TGT GAC CGC TTC TGC CGT
     Leu Tyr Ser Thr Pro Ser Glu Asn Leu Cys Asp Arg Phe Cys Arg  555
1852 CTG GAT ACC GCC GAG TTT GGC GTG GTG CCG GGC GTA ACC GAT AAA
     Leu Asp Thr Ala Glu Phe Gly Val Val Pro Gly Val Thr Asp Lys  570
1897 GGT TAC TAC ACC AAC AGC TTC CAC CTC GAC GTG GAG AAG AAG GTC
     Gly Tyr Tyr Thr Asn Ser Phe His Leu Asp Val Glu Lys Lys Val  585
1942 AAC CCG TAC GAC AAA ATC GAT TTC GAA GCG CCG TAT CCG CCG CTG
     Asn Pro Tyr Asp Lys Ile Asp Phe Glu Ala Pro Tyr Pro Pro Leu  600
1987 GCG AAC GGG GGT TTC ATT TGC TAC GGC GAA TAC CCG AAC ATT CAG
     Ala Asn Gly Gly Phe Ile Cys Tyr Gly Glu Tyr Pro Asn Ile Gln  615
2032 CAC AAC CTG AAA GCG CTG GAA GAT GTC TGG GAT TAC AGC TAT CAG
     His Asn Leu Lys Ala Leu Glu Asp Val Trp Asp Tyr Ser Tyr Gln  630
2077 CAT GTA CCG TAT TAC GGC ACC AAC ACG CCG ATC GAT GAA TGC TAC
     His Val Pro Tyr Tyr Gly Thr Asn Thr Pro Ile Asp Glu Cys Tyr  645
2122 GAG TGC GGC TTT ACC GGG GAG TTC GAA TGT ACC AGT AAA GGT TTC
     Glu Cys Gly Phe Thr Gly Glu Phe Glu Cys Thr Ser Lys Gly Phe  660
                    nrdcdlp2rev
                    <-------
                    ------->
                    nrdcdlp2
2167 ACC TGC CCG AAA TGC GGC AAC CAC GAC GCC GCG CGC GTC TCG GTC
     Thr Cys Pro Lys Cys Gly Asn His Asp Ala Ala Arg Val Ser Val  675
2212 ACC CGC CGC GTG TGC GGT TAT TTA GGC AGC CCG GAC GCG CGT CCG
     Thr Arg Arg Val Cys Gly Tyr Leu Gly Ser Pro Asp Ala Arg Pro  690
2257 TTT AAC GCC GGT AAG CAG GAA GAA GTG AAG CGC CGC GTG AAG CAT
     Phe Asn Ala Gly Lys Gln Glu Glu Val Lys Arg Arg Val Lys His  705
2302 TTA GGC AAT GGG CAG ATA GGT TAA
     Leu Gly Asn Gly Gln Ile Gly Stop                            712
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Fig. 3.6 (cont'd)

Fig.3.6 The complete nucleotide sequence and predicted amino acid sequence of *S. typhimurium nrdDG*. The arrows indicate the position and orientation of the primers used in sequencing. REP: Repetitive extragenic palindromic sequence

3.4 Discussion

3.4.1 Sequence analysis of *S. typhimurium nrdDG.*

The nucleotide sequence of the 5' untranslated region of *S. typhimurium nrdDG* was determined (Wong, 1990). In this project, the coding and the 3' untranslated region of the operon were sequenced. This operon contains two ORFs with a short intervening sequence. The sequence determined in this project started from the 1308 bp of the insert of pFSH8, which is a clone carrying a 2 kb genomic fragment of *S. typhimurium* showing homology to the *E. coli nrdD* (Wong, 1990). Several discrepancy were identified between pFSH8 and sequence determined in this experiment. The correct sequence was confirmed by direct sequencing of PCR prodcut amplified from pFSH8, genomic DNA as well as the lambda clones. There were single base insertions in position 1429(T), 1431(C), 1438(A) and 1447(A) of pFSH8. An inversion was found in position 1579-1580 of pFSH8, which should be GC instead of CG. The pFSH8 sequence also contained a 3 bp (CCA) deletion in position 1625. Two consecutive missense mutations were found in position 1948-50 and 1951-53. The first one should be CAA (Gln) instead of CAC (His), the second one should be GCG (Ala) instead of ACG (Thr). These errors were probably due to unclear bands generated in manual sequencing. The corrected sequence is shown as Fig. 3.7.

The RNA polymerase holoenzyme will recognize specific consensus region at promoter regions (Hawley and McClure, 1983; Harley and Reynolds, 1987; Lisser and Margalit, 1993). The two hexameric elements located at -35 and -10 relative to the transcriptional start point (+1) have consensus sequence TTGACA and

TATAAT respectively. They are usually separated by a spacer of 15 to 21 bp. As shown in Fig. 3.7, independent -35 or -10 consensus sequence are scattered in the putative promoter region of *nrdDG* of *S. typhimurium* but they are not organized with a defined spacer region in a consensus manner. The absence of consensus -35 or -10 sequence may indicate that the gene may be under control of other elements such as upstream (UP) elements (Ross *et al.*, 1993). The precise transcriptional start point and the upstream regulatory sequences are still needed to be determined.

A putative ribosomal binding site (GGAG) was identified 5 bp upstream of the putative translational start codon ATG of the first ORF. This site is complementary to the 3' end of 16S rRNA of *S. typhimurium* (Cilia *et al.*, 1996), and is important for ribosome to bind to the mRNA and initiate the protein synthesis.

Fnr and ArcA are two major global regulators that regulate the expression of many genes involved in anaerobic metabolism of enteric bacteria (Guest *et al.*, 1996; Lynch and Lin, 1996a). They exert their effect by binding on a specific sequence of the promoter region. Fnr binds to DNA containing partially palindromic consensus sequences TTGAT----ATCAA (Bell *et al.*, 1989; Eiglmeier *et al.*, 1989). The 28 dyad symmetry Fnr binding site G--AAA-TTGAT----ATCAA-TTT--C (Eiglmeier *et al.*,1989) was found in the pFSH8 sequence at position 1417 to 1444 (Wong, 1990). However, since sequencing errors were found at this site, the conserved dyad symmetry vanished in the corrected sequence. On the other hand, base on the partial palindromic sequence TTGAT----ATCAA, two putative Fnr binding sites were identified in the corrected promoter region. The first one is located at position 1245 to 1258 of pFSH8 (TTGTT----ATCAA), the other is located at position 1276 to

1289 (TTGAT----ATCAC), both of them possess only 1 bp mismatch to the consensus sequence. Consecutive Fnr binding site was also identified in many Fnr regulated genes of *E. coli* such as *ndh* (Green and Guest, 1994). Presence of Fnr binding site in the promoter of *nrdDG* gene *of S. typhimurium* suggests that this gene may be regulated by Fnr.

Recently, the consensus binding sequence of phosphorylated ArcA was identified (Lynch and Lin, 1996b). It is a 10 bp consensus sequence [A/T]GTTAATTA[A/T] which could be found in position 1188 of the pFSH8 with 2 bp mismatch (TGTTGAATAA). The presence of both Fnr and ArcA binding site in the promoter region *of S. typhimurium nrdDG* operon suggests that this operon is regulated by oxygen. Regulation of *nrdDG* gene *of S. typhimurium* will be discussed in the next chapter.

putative ArcA binding site

```
  1 CCTATTGACA CGGGCTGTGG CGTTGCTCCA GGTTACTGAC TGTTTTTGTT GAATAATTAA
 61 TCAGATTTTT CTTGCTATCT TTAACAATGC ACAGCGCCGC CATCTGTAGC TCTGATTTTT
        putative Fnr binding site                 putative Fnr binding site
121 ACCTTGTTCT ACATCAATAA AATTGCAAAC ATCCTTGATG CAAATCACTA CATATAGACT
181 TTAAAATGCA CACCGACCCA ATATGTTGTA TTAATTGACT ACAATTGCTA CAACACCTGT
241 TCACTCGACA CAAGGTGAAT TGTGGATAAC CTGGGTCAGG ATTGCGGGAA GTCGTTGGAA
301 AAGAGATGAA TAAACCTGTT ATGGCTTCCC CGGCCTCTGT GGATAACCTG TTCTTACAAA
       SD       MET
361 TATGGAGTGA TCATGACACC GCATGTGATG AAACGAGATG GCTGTAAAGT GCCATTCAAA
421 TCAGAGCGCA TTAAGGAAGC CATTCTACGT GCAGCTAAAG CAGCGGGAGT CGATGACGCA
481 GATTACTGTG CCACCGTCGC AGAAGTCGTT AGCAGCCAAA TGAACGCGCG CAGTCAGGTG
541 GATATTAACG AGATCCAAAC TGCGGTTGAA AACCAACTGA TGTCCGGCCC GTACAAACAG
601 CTTGCCCGCG CCTACATCGA ATACCGTCAC GATCGCGACA TTCAGCGTGA AAAGCGTGGT
661 CGTCTGAACC AGGAAATTCG CGGCCTGGTA GAGCAAACTA ACTCCGCGTT GCTCAATGAA
721 AACGCCAACA AAGACAGTAA AGTCATTCCC ACCCAGCGCG ATTTGCTGGC CGGGATTGTC
781 GCCAAACACT ATGCCCGCCA GCACCTGTTG CCGCGTGACG TAGTACAAGC GCATGAGCGC
841 GGCGATATCC ACTATCACGA TC
```

Fig. 3.7 Corrected pFSH8 sequence and putative important features

By searching the NCBI GenBank using BLASTX, the first ORF, which corresponds to *nrdD* of *S. typhimurium*, matched several ribonucleotide reductases. It matched the anaerobic ribonucleotide reductase (NrdD) of *E. coli* (Sun *et al.*, 1993) at the highest homology (93% identities and 95% positive). The homology is throughout the entire protein sequence (Fig. 3.8a). Several important domains, though uncharacterized, were identified in NrdD of *E. coli.* Firstly, the C-terminal of the protein possesses a high cysteine content (-Cys-Xaa_2-Cys-Xaa_2-Cys-Xaa_{14}-Cys-), these cysteine residues are all conserved in the *Salmonella* protein. This domain was first thought to be an Iron-sulfur cluster (Sun *et al.*, 1993; Mulliez *et al.*, 1993), however, the iron-sulfur cluster was later found in another part of the reductase (Ollagnier *et al.*, 1996). Nevertheless, protein sequence rich in cysteine content may suggest this is an important domain for the protein structure. If this domain actually plays a role in the catalysis of ribonucleotide reduction, this role would be conserved in *S. typhimurium*. Another important feature of the *E. coli* protein is the glycine residue at position 681. Since the amino acid sequence flanking glycine is identical to the pyruvate formate lyase (Pfl) of *E. coli*, it is proposed that Pfl and nrdD share the similar mechanism for catalysis (Sun *et al.*, 1993). This amino acid residue will change to a glycyl radical when the enzyme is activated (King and Reichard, 1995; Sun *et al.*, 1993; Sun *et al.*, 1996). The glycine residue and the surrounding amino acid of the *Salmonella* protein are also conserved, therefore, this enzyme would be activated in the same manner. The activating protein was also identified in *S. typhimurium* (See discussion below). Since the overall protein sequence of NrdD of *E. coli* and *S. typhimurium* is highly homologous, I propose that the reaction mechanisms such as

co-factor requirement, substrate specificity and allosteric regulation of the protein are similar in both species. Experimental evidence is needed to support this hypothesis.

Several microbial genomes were already completely sequenced. Anaerobic ribonucleotide reductase homologues were found in two of them. The first one is in the genome of *Haemophilus influenzae* Rd (Fleischmann *et al.*, 1995) which showed 88% similarity to *E. coli* NrdD. The other is located in the genome of a methanogenic archaeon *Methanococcus jannaschii* (Bult *et al.*, 1996) which showed 28% identity to the *E. coli* protein. This protein homologue carry two putative inteins. Both *H. influenzae* Rd and *M. jannaschii* homologues also showed homology to the *S. typhimurium* NrdD with 72% and 40% identity respectively. The presence of the NrdD homologues in the genomes of *H.influenzae* and *M. jannaschii* suggests that this enzyme is important for the anaerobic life style of bacteria. The genome of obligate anaerobe *M. jannaschii* possess only anaerobic but not the aerobic ribonucleotide reductase homologue suggesting that the bacteria depend solely on this enzyme for anaerobic reduction of ribonucleotides although other reductase might missed during annotation of the genome sequence (Bult *et al.*, 1996). However, in the genome of *H. influenzae* Rd (Fleischmann *et al.*, 1995), an aerobic as well as an extra anaerobic ribonucleotide reductase are present, the latter can be responsible for ribonucleotide reduction in anaerobic environment. This redundancy would allow the bacteria to adapt to the facultative anaerobic metabolism. This postulation can be applied in *S. typhimurium* since two aerobic ribonucleotide reductases were identified in *S. typhimurium* (Jordan *et al.*, 1994 a and b; Jordan *et al.* 1995), the presence of the

anaerobic ribonucleotide reductase indicates that the bacteria would use a different enzyme for ribonucleotide reductase in the anaerobic environment.

The *Salmonella* enzyme also showed homology to the ribonucleotide reductases of two archaebacteria *Thermoplasma acidophila* (Riera *et al.* ,1997) and *Pyrococcus furiosus* (Tauer and Benner, 1997). The homology were restricted at the N-terminal of the protein only. Moreover, this homology extends to the aerobic enzyme (Fig. 3.8b). The ribonucleotide reductases of two archaebacteria were proposed be the closest candidate to the ancestral protein which give rise to different ribonucleotide reductase by divergent evolution (Riera *et al.* ,1997; Tauer and Benner, 1997). However, anaerobic ribonucleotide reductase was also proposed to be the ancestral protein (Reichard, 1997). No matter whether one of them is the true ancestral protein, the conservation of this N-terminal domain among evolutionary distant species suggest that this domain play an indispensible role in ribonucleotide reduction.

A 120 bp non-coding sequence was found preceeding the second ORF. In this sequence, no consensus sequence related to promoter sequence can be identified. Instead, there are two extragenic repetitive palindromic sequences (REP, Stern *et al.*, 1984). Therefore, the downstream ORF is organized with the upstream ORF to form an operon under control of the same promoter. This organization is also found in the *nrdDG* operon of *E. coli* (Sun *et al.*, 1993; Sun *et al.*, 1995).

The second ORF matched anaerobic ribonucleotide reductase activating protein (NrdG) of *E. coli* (Sun, 1995), *H. influenzae* (Fleischmann *et al.*, 1995), and bacteriophage T4 (Young *et al.*, 1994b). At the same time, this ORF matched

pyruvate formate-lyase activating enzyme (PflA) of several organisms such as *E. coli* (Rödel *et al.*, 1988;), *H. influenzae* (Fleischmann *et al.*, 1995) and *Clostridium pasteurianum* (Weidner and Sawers, 1996). NrdG and PflA are functionally different, but three conserved cysteine residues are found on their N-terminal amino acids sequence (Fig. 3.8c). These residues are important for the pyruvate formate-lyase (Pfl) activation in *E. coli*. Pfl was activated by the generation of a glycyl radical inside the enzyme through the action of PflA. The conserved cysteines residues were suggested to bind iron to form an Fe-S cluster which is important to generate the radical (Wagner *et al.*, 1992). The same mechanism was proposed for activation of anaerobic ribonucleotide reductase (Sun *et al.*, 1995). However, in instead of existing as a separate enzyme, the activating enzyme of ribonucleotide reductase of *E. coli* form a homodimer and bind to the homodimer of the ribonucleotide reductase subunit to form an active protein (Ollagnier *et al.*, 1996). The overall similarity of *S. typhimurium* anaerobic ribonucleotide reductase to *E. coli* suggests that they share the same structural properties and reduction mechanism for ribonucleotide reduction. This postulation can be verified by overexpressing a recombinant NrdDG of *S. typhimurium* and characterize its biochemical properties.

3.4.2 Experimental design.

Once a lambda clone containing an insert of interest is isolated, the traditional approach is to determine the restriction map of the clone and subclone the restriction fragments that contain the gene. My approach to sequence the *nrdDG* genes of *S. typhimurium* depended on PCR amplification with one gene specific primer and one

vector specific primer. The gene specific primer (nrdDup2) was designed according to the sequence of pFSH8, which contained an *S. typhimurium* genomic fragment that showed homology to the 5' untanslated region of *E. coli nrdD* gene (Wong, 1990). The nrdDup2 primer should anneal to the promoter region of *nrdDG* of *S. typhimurium* while the other end was flanked by the vector specific primer. Since the orientation of the insert was not known, both T3 and T7 were tested. Either T3 or T7 primers could successfully amplify the *nrdDG* gene from different lambda clones with the gene specific primer. Long distance PCR was used for the amplification to ensure that a reasonable sized PCR product could be obtained to increase the chance of getting the complete gene. LD-PCR products ranged from 2.3-10 kb were obtained, all except the 2.3 kb fragment were longer than the *nrdDG* operon of *E. coli.* It was subsequently shown that these clones carry the full length fragment of the gene.

Both PCR amplified DNA and plasmid DNA can be used as template for DNA sequencing. In my experiment, both LD-PCR products and recombinant plasmids were used. Subcloning provided two chimeric ends that can be used for primer annealing for the sequencing reaction so that time was saved. However, since *Taq* polymerase does not possess 100% fidelity in DNA synthesis (Keohavong and Thilly, 1989), PCR products are in fact a mixture of DNA fragments of which a proportion carry mutation. Subcloning PCR product may separate these mutated sequences from the mixture and introduce an error on sequencing. However, if more than one clone is being sequenced, the consensus and the majority sequence can be revealed. Therefore, in the sequencing experiment, for each clone carried the same insert, I had analyzed three individual isolates in both strands. Moreover, I also

performed direct sequencing of LD-PCR product which gave a definite sequence since the error in any one position will be masked by the majority in the mixture (Andersson and Gibbs, 1994).

```
StyNrdD:   187 MTPHVMKRDGCKVPFKSERIKEAILRAAKAAGVDDADYCATVAEVVSSQMNARSQVDINE 366
               MTPHVMKRDGCKVPFKSERIKEAILRAAKAA VDDADYCATVA VVS QM  R+QVDINE
EcoNrdD:     1 MTPHVMKRDGCKVPFKSERIKEAILRAAKAAEVDDADYCATVAAVVSEQMQGRNQVDINE 60

StyNrdD:   367 IQTAVENQLMSGPYKQLARAYIEYRHDRDIQREKRGRLNQEIRGLVEQTNSALLNENANK 546
               IQTAVENQLMSGPYKQLARAYIEYRHDRDI+REKRGRLNQEIRGLVEQTN++LLNENANK
EcoNrdD:     61 IQTAVENQLMSGPYKQLARAYIEYRHDRDIEREKRGRLNQEIRGLVEQTNASLLNENANK 120

StyNrdD:   547 DSKVIPTQRDLLAGIVAKHYARQHLLPRDVVQAHERGDIHYHDLDYSPFFPMFNCMLIDL 726
               DSKVIPTQRDLLAGIVAKHYARQHLLPRDVVQAHERGDIHYHDLDYSPFFPMFNCMLIDL
EcoNrdD:    121 DSKVIPTQRDLLAGIVAKHYARQHLLPRDVVQAHERGDIHYHDLDYSPFFPMFNCMLIDL 180

StyNrdD:   727 KGMLTQGFKMGNAEIEPPKSISTATAVTAQIIAQVASHIYGGTTINRIDEVLAPFVTESY 906
               KGMLTQGFKMGNAEIEPPKSISTATAVTAQIIAQVASHIYGGTTINRIDEVLAPFVT SY
EcoNrdD:    181 KGMLTQGFKMGNAEIEPPKSISTATAVTAQIIAQVASHIYGGTTINRIDEVLAPFVTASY 240

StyNrdD:   907 NKHRKTADEWQIPDAEGYARSRTEKECYDAFQSLEYEVNTLHTANGQTPFVTFGFGLGTS 1086
               NKHRKTA+EW IPDAEGYA SRT KECYDAFQSLEYEVNTLHTANGQTPFVTFGFGLGTS
EcoNrdD:    241 NKHRKTAEEWNIPDAEGYANSRTIKECYDAFQSLEYEVNTLHTANGQTPFVTFGFGLGTS 300

StyNrdD:  1087 WESRLIQASILRNRIAGLGKNRKTAVFPKLVFAIRDGLNHKFGDPNYDIKQLALECASKR 1266
               WESRLIQ SILRNRIAGLGKNRKTAVFPKLVFAIRDGLNHK GDPNYDIKQLALECASKR
EcoNrdD:    301 WESRLIQESILRNRIAGLGKNRKTAVFPKLVFAIRDGLNHKKGDPNYDIKQLALECASKR 360

StyNrdD:  1267 MYPDILNYDQVVKVTGSFKTPMGCRSFLGVWENENGEQIHDGRNNLGVISLNLPRIALEA 1446
               MYPDILNYDQVVKVTGSFKTPMGCRSFLGVWENENGEQIHDGRNNLGVISLNLPRIALEA
EcoNrdD:    361 MYPDILNYDQVVKVTGSFKTPMGCRSFLGVWENENGEQIHDGRNNLGVISLNLPRIALEA 420

StyNrdD:  1447 KGDETAFWKLLDERLALARKALMTRIARLEGVKARVAPILYMEGACGVRLKADDDVSEIF 1626
               KGDE  FWKLLDERL LARKALMTRIARLEGVKARVAPILYMEGACGVRL ADDDVSEIF
EcoNrdD:    421 KGDEATFWKLLDERLVLARKALMTRIARLEGVKARVAPILYMEGACGVRLNADDDVSEIF 480

StyNrdD:  1627 KNGRASISLGYIGIHETINALFGGEHLYDSEQLRAKGIAIVERLRQAVDQWKDETGYGFS 1806
               KNGRASISLGYIGIHETINALFGGEH+YD+EQLRAKGIAIVERLRQAVDQWK+ETGYGFS
EcoNrdD:    481 KNGRASISLGYIGIHETINALFGGEHVYDNEQLRAKGIAIVERLRQAVDQWKEETGYGFS 540

StyNrdD:  1807 LYSTPSENLCDRFCRLDTAEFGVVPGVTDKGYYTNSFHLDVEKKVNPYDKIDFEAPYPPL 1986
               LYSTPSENLCDRFCRLDTAEFGVVPGVTDKGYYTNSFHLDVEKKVNPYDKIDFEAPYPPL
EcoNrdD:    541 LYSTPSENLCDRFCRLDTAEFGVVPGVTDKGYYTNSFHLDVEKKVNPYDKIDFEAPYPPL 600

StyNrdD:  1987 ANGGFICYGEYPNIQHNLKALEDVWDYSYQHVPYYGTNTPIDECYECGFTGEFECTSKGF 2166
               ANGGFICYGEYPNIQHNLKALEDVWDYSYQHVPYYGTNTPIDECYECGFTGEFECTSKGF
EcoNrdD:    601 ANGGFICYGEYPNIQHNLKALEDVWDYSYQHVPYYGTNTPIDECYECGFTGEFECTSKGF 660

StyNrdD:  2167 TCPKCGNHDAARVSVTRRVCGYLGSPDARPFNAGKQEEVKRRVKHLGNGQIG 2322
               TCPKCGNHDA+RVSVTRRVCGYLGSPDARPFNAGKQEEVKRRVKHLGNGQIG
EcoNrdD:    661 TCPKCGNHDASRVSVTRRVCGYLGSPDARPFNAGKQEEVKRRVKHLGNGQIG 712
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StyNrdG:   121 MRYHQYYPVDIVNGPGTRCTLFVSGCVHECPGCYNKSTWRLNSGQPFTKEMEDKIIADLN 300
               M YHQYYPVDIVNGPGTRCTLFVSGCVHECPGCYNKSTWR+NSGQPFTK MED+II DLN
EcoNrdG:     1 MNYHQYYPVDIVNGPGTRCTLFVSGCVHECPGCYNKSTWRVNSGQPFTKAMEDQIINDLN 60

StyNrdG:   301 DTRIHRQGISLSGGDPLHPQNVPDILALVQRIHAECPGKDIWVWTGYKLDKLNAAQMQVV 480
               DTRI RQGISLSGGDPLHPQNVPDIL LVQRI AECPGKDIWVWTGYKLD+LNAAQMQVV
EcoNrdG:    61 DTRIKRQGISLSGGDPLHPQNVPDILKLVQRIRAECPGKDIWVWTGYKLDELNAAQMQVV 120

StyNrdG:   481 DLINVLVDGKFVQDLKNPALIWRGSSNQVVHHLR 582
               DLINVLVDGKFVQDLK+P+LIWRGSSNQVVHHLR
EcoNrdG:   121 DLINVLVDGKFVQDLKDPSLIWRGSSNQVVHHLR 154
```

Fig. 3.8a Upper panel: Homology between *E. coli* anaerobic ribonucleotide reductase amino acid sequence (EcoNrdD) and *S. typhimurium* anaerobic ribonucleotide reductase amino acid sequence (StyNrdD). Lower panel: Homology between *E. coli* anaerobic ribonucleotide reductase activating enzyme amino acid sequence (EcoNrdG) and *S. typhimurium* anaerobic ribonucleotide reductase activating enzyme amino acid sequence (StyNrdG).

```
StyNrdD:    MTPHVMKRDGCKVP
EcoNrdD:    MTPHVMKRDGCKVP
TacNrd :    MIKEVVKRDGTVVP
PfuNrd :   MAVEKVMKRDGRIVP
EcoNrdA:      MHVIKRDGRQER
Common:       --V-KRDG----
```

Fig. 3.8b Homology between the amino acid sequences of different classes of ribonucleotide reductases.
StyNrdD: anaerobic ribonucleotide reductase of *S. typhimurium.*
EcoNrdD: anaerobic ribonucleotide reductase of *E. coli.*
TacNrd: ribonucleotide reductase of *Thermoplasma acidophila.*
PfuNrd: ribonucleotide reductase of *Pyrococcus furiosus.*
EcoNrdA: ribonucleotide reductase of *E. coli* (aerobic form).

```
StyNrdG: MRYHQYYPVDIVNGPGTRCTLFVSGCVHECPGCYNKSTWRLNSGQPFTKE
EcoNrdG: MNYHQYYPVDIVNGPGTRCTLFVSGCVHECPGCYNKSTWRVNSGQPFTKA
HinNrdG: MNYLQYYPTDVINGEGTRCTLFVSGCTHACKGCYNQKSWSFSAGVLFDDV
T4NrdG : MNYDRIYPCDFVNGPGCRVVLFVTGCLHKCEGCYNRSTWNARNGQLFTMN
EcoPflA:  RIHSFESCGTVDGPGIRFITFFQGCLMRCLYCHNRDTWDTHGGKEVTVE
HinPflA:  RIHSFESCGTVDGPGIRFITFFQGCLMRCLYCHNRDTWDTHGGKEVTVE
CpaPflA:  RIHSIESMGLVDGPGIRTVVFFQGCGLRCSYCHNPDTWNMAGGKELTAE

common :    ----------G-G-R---F--GC---C--C-N---W----G------
```

Fig. 3.8c Homology between the amino acid sequences of ribonucleotide reductase activating enzyme and pyruvate formate-lyase activating enzyme. StyNrdG: anaerobic ribonucleotide reductase activating enzyme of *S. typhimurium.* EcoNrdG: anaerobic ribonucleotide reductase activating enzyme of *E. coli.* HinNrdG: anaerobic ribonucleotide reductase activating enzyme of *Haeomphilus influenzae*. T4NrdG: anaerobic ribonucleotide reductase activating enzyme of bacteriophage T4. EcoPflA: pyruvate formate-lyase activating enzyme of *E. coli.* HinPflA: pyruvate formate-lyase activating enzyme of *H.influenzae*.. CpaPflA: pyruvate formate-lyase activating enzyme of *Clostridium pasteurianum.*

Chapter 4

Transcriptional regulation of anaerobic ribonucleotide reductase in *Salmonella typhimurium* in aerobic and anaerobic environments.

4.1 Introduction

In this chapter, expression of anaerobic ribonucleotide reductase (NrdD) in *S. typhimurium* in both aerobic and anaerobic environments was investigated. NrdD of *E. coli* is an oxygen-sensitive enzyme, upon exposure to oxygen, the enzyme lose its function (Fontecave *et al.*, 1989). The loss of function is probably due to truncation of C-terminal of the protein at a glycine radical (King and Reichard, 1995; Sun *et al.*, 1993). If there is no regulation of enzyme production, it would be a great wastage to the bacterial cell. Furthermore, the transcription of aerobic ribonucleotide reductase (NrdAB) of *E. coli* was found to be down-regulated under anaerobiosis (Casado *et al.*, 1991), suggesting that the *nrdD* would also be under transcriptional regulation. In addition, putative binding sites for global transcriptional regulators Fnr and ArcA were identified in the 5' untranslated region of *nrdD* of *E. coli* (Sun *et al.*, 1993) and *S. typhimurium* (Wong, 1990 and chapter 3). Presence of putative binding sites for these regulators suggests that this enzyme is regulated by these factors. Fnr and ArcA are well documented regulator for anaerobic metabolism of enteric bacteria (Guest *et al.*, 1996; Lynch and Lin, 1996b), therefore, *nrdD* may also be regulated by oxygen availability. A ten-fold increase in the expression of *nrdD* in anaerobic environment in *S. typhimurium* was found by using an episomal *lacZ* fusion on a low copy number plasmid (Wong, 1990).

Regulation of transcription of *nrdD* has not yet been well documented in enteric bacteria. In this study, both reverse transcriptase polymerase chain reaction (RT-PCR) and Northern blot hybridization were used to investigate the transcript levels of *nrdD* in *S. typhimurium* under different growth conditions. The measurement of transcripts eliminates the concern of copy numbers when episomal *lacZ* fusions are tested. The effect of *fnr* , formerly known as *oxrA* in *S. typhimurium*, was also investigated by RNA dot blot analysis.

4.2 Materials and methods

4.2.1 Bacteria and bacteriophage strain.

S. typhimurium wild type LT2 and its derivative TN2336 (*leuBCD485 pepT7::MudJ oxrA2::Tn10*) (C. G. Miller, Personal communication) were used throughout the experiment.. P22 HT *int* was used for transduction.

4.2.2 Culture media.

LBE medium was LB medium (3.2.2) buffered with 1X NCE medium.

1X NCE medium was diluted from a 50 X NCE stock which consisted of ,per liter, 197 g KH_2PO_4 (potassium phosphate monobasic), 323 g $K_2HPO_4 \cdot 3H_2O$ (potassium phosphate dibasic) and 175 g $NaNH_4HPO_4 \cdot 4H_2O$ (sodium ammonium phosphate.

P22 broth was prepared as 100:2:1 of NB, E medium (50X), 20% glucose solution. NB is 0.8% nutrient broth (Difco). E medium (50 X) consisted of, per liter, 10 g $MgSO_4 \cdot 7H_2O$, 100 g citric aicd.$1H_2O$ (granular), 655 g $K_2HPO_4 \cdot 3H_2O$ (potassium phosphate dibasic) and 175 g $NaNH_4HPO_4 \cdot 4H_2O$ (sodium ammonium phosphate).

MacConkey-nitrate medium (Barrett *et al.*, 1979) consisted of 40 g MacConkey agar base, 0.5 g NaCOOH, 0.5 g glycerol, 0.1 g glucose and 10 g KNO_3 per liter. All components were added from sterile 20 X stock solutions to the autoclaved agar.

Antibiotics were added from stock solutions to the autoclaved medium when needed. Ampicillin and tetracycline were added at 100 μg/ml and 15 μg/ml respectively.

4.2.3 Construction and characterization of *oxrA* mutant

4.2.3.1 Preparation of P22 lysate of TN2336.

P22 lysates were grown as described by Maloy (1990). Strain TN2336 was grown overnight in NB at 37°C with shaking. one ml overnight culture was added with 4 ml P22 broth together with P22 phage at multiplicity of infection (moi) of 0.01-0.1 pfu/cell. The mixture was grown for 8 to16 hours at 37°C with shaking. The bacterial debris was removed by centrifugation and the lysate was kept at 4 °C with a few drops of chloroform.

4.2.3.2 P22 transduction for construction of *oxrA* mutant.

LT2 was grown overnight in NB at 37°C with shaking. This culture (0.1 ml) was mixed with 0.05-0.2 ml P22 lysate of TN2336 on a LB plate with tetracycline to select for transductants with *oxrA::Tn10*. The plate was incubated at 37°C overnight. Overnight LT2 culture and P22 lysate were plate separately on LB plates with tetracycline as negative controls.

4.2.3.3 Characterization of *oxrA* mutant.

Transductants from the above transduction were streaked on MacConkey-nitrate agar plates and incubated at 37°C overnight anaerobically in an anaerobic jar filled with H_2 and CO_2. Under this condition, the *oxrA* mutant forms a tiny dark red colonies while the wild type appeared as large white colonies.

4.2.4 Extraction of bacterial RNA by hot phenol method.

Total RNA from *S. typhimurium* was extracted by the hot phenol method as described by Wong and McClelland (1994). LBE (with antibiotic as necessary) was inoculated with overnight culture at 1%. For aerobic condition, the cultures were shaken at 200 rpm at 37°C. For anaerobic condition, cultures were grown in test tubes with medium filled up to the top. The test tubes were plugged with stoppers and incubated at 37°C without shaking. To harvest cells, 10-15 ml culture at log phase was centrifuged at 4800 rpm at 4°C for 10 minutes. The bacterial pellet was suspended in 0.5 ml 30 mM sodium acetate(pH 5.2). The suspension was transferred to an eppendorf tube, 50 µl 20% SDS and 0.5 ml saturated phenol (pH 5.2) was added to the suspension. The mixture was vortexed, incubated at 65 °C for 10 minutes and shaken every 2 minutes. Then, the mixture was centrifuged at 13000 rpm at the room temperature for 5 minutes. The upper aqueous phase was retained and extracted with phenol at 65°C twice, and with chloroform twice at room temperature. RNA was precipitated by adding 1/4 volume 3M sodium acetate (pH 5.2) together with 3 volume 100% ethanol and kept at -20°C for 1 hour. RNA was precipitated by centrifugation at 13000 rpm at 4°C for 10 minutes. The RNA pellet was washed with 70% ethanol, dried and resuspended in 100 µl DEPC-treated ultrapure water.

4.2.5 Formaldehyde gel electrophoresis of RNA

RNA samples were resolved by 1.2% denaturing formaldehyde agarose electrophoresis in 1X formaldehyde running buffer which contained 0.1 M MOPS, 40 mM sodium acetate and 5 mM EDTA, pH 8.0. The 1.2% agarose contained 1X

formaldehyde running buffer and 6% formaldehyde. Before electrophoresis, the agarose gel was first pre-run for 5 minutes at 5V/cm. To each 4.5 µl RNA sample, 2 µl 5X formaldehyde running buffer, 10 µl formamide, 3.5 µl 37% formaldehyde and 0.8 µg ethidium bromide were added. The mixture was heated at 65°C for 10 minutes and electrophoresed at 3V/cm.

4.2.6 Reverse transcriptase polymerase chain reaction (RT-PCR) of *nrdD* transcript.

RNA preparations were treated with DNase I treatment to digest any DNA contaminant by adding 1 unit of DNase I (Gibco BRL) to 1 µg of RNA in 1 X DNase I reaction buffer (20 mM Tris-HCl [pH 8.4], 2 mM $MgCl_2$, 50 mM KCl) in 10 µl reaction volume at room temperature for 15 minutes. Afterwards, DNase I was inactivated by adding 1 µl 25 mM EDTA and heated at 65°C for 10 minutes. The treated RNA was used for RT-PCR.

One µg DNase I treated RNA was mixed with 2 pmoles of primer *nrdD*lp2 in 12 µl volume, incubated at 70°C for 10 minutes and then chilled on ice. Four µl 5X First strand buffer (250 mM Tris-HCl [pH 8.3], 375 mM KCl and 15 mM $MgCl_2$), 2 µl 0.1 M DTT and 1 µl 10 mM dNTPs mix (10 mM each dATP, dGTP, dCTP and dTTP) were added to the RNA/primer mixture and incubated at 42°C for 2 minutes. One µl (200 units) SUPERSCRIPT™ RNase H^- Reverse Transcriptase (Gibco BRL) was then added to the mixture and incubated at 42°C for 50 minutes. The enzyme was inactivated by heating at 70°C for 15 minutes. cDNA synthesized was then used

for PCR amplification. A control with no reverse transcriptase added was included to ensure that no false positives were generated by DNA contamination.

PCR amplification was performed in 25 µl which contained 1X PCR buffer (Promega , 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 1% Triton® X-100), 2 mM $MgCl_2$, each 200 µM dNTPs, 400 nM primer *nrdD*up1 (TGACTGTTTTTGTTGAATAA, bp 37 of Fig. 3.7), 400 nM primer *nrdD*lp1 (GCCATCTCGTTTCATCACAT, 5' end complementary to bp 402 of Fig.3.7), 2 µl cDNA from the RT reaction and 0.625 units of *Taq* DNA polymerase (Promega). The PCR profile consisted of 1 cycle of denaturation at 80 °C for 5 minutes, then 30 cycles of denaturation at 94 °C for 1 minute, primer annealing at 50 °C for 1 minute and elongation at 72 °C for 1 minute. PCR products were sampled at every 5 cycles of amplification. The RT-PCR product was resolved on 1.5% agarose gel electrophoresis in 1X TBE buffer.

4.2.7 Transfer of DNA/RNA to solid support.

4.2.7.1 Transfer of DNA to solid support by Southern blotting

After electrophoresis of DNA samples, the agarose gel was placed in 0.25 M HCl until the dye in the loading buffer had changed colour and then left for 10 more minutes. The gel was rinsed with distilled water and placed in denaturation buffer (1.5 M NaCl, 0.5 M NaOH) for 30 minutes. The gel was rinsed again with distilled water and placed in neutralization buffer (1.5 M NaCl, 0.5 M Tris-HCl [pH 7.2] and 0.001 M EDTA) twice, each for 15 minutes. Then a capillary blot was set up as follow. A glass tray was filled

with 20X SSPE (3.6M NaCl, 0.2M sodium phosphate and 0.02M EDTA). A filter paper wick saturated with 20X SSPE was placed on a platform. The two ends of the wick were submerged into 20X SSPE in the glass tray. The treated agarose gel was placed on the wick and air bubbles between the gel and the wick were removed. Parafilm strips were used to surround the gel. A sheet of nylon membrane (Hybond-N, Amersham) was cut to the size of the gel, placed above the gel, and the air bubbles between the gel and the membrane were removed. Three sheets of Whatman 3 MM filter paper was cut to the size of the membrane, wetted with 20X SSPE, and place on top of the nylon membrane. A stack of paper towels was placed on the Whatman 3 MM paper. A glass plate was placed on top of the stack of paper towel. Then a 0.75-1 kg weight was put on the glass plate. The blotting was allowed to proceed for at least 12 hours. After blotting, the membrane was washed in 2X SSPE and air dried. DNA on the membrane was fixed by UV crosslinking.

4.2.7.2 Transfer of RNA to solid support by Northern blotting

RNA was resolved by denaturing formaldehyde agarose gel electrophoresis. After electrophoresis, the gel was rinsed in DEPC-treated water and used for blotting directly. A capillary blot was set up as described in section 4.2.7.1. RNA was fixed to the membrane by UV crosslinking.

4.2.7.3 RNA dot blot.

RNA was directly transferred to solid support by Bio-dot® Microfiltration Apparatus (Bio-Rad). The apparatus was cleaned and dried prior to use. The gasket support plate was first fitted into the vacuum

manifold, the sealing gasket was then aligned and place on top of the support plate. A piece of size-fitted Hybond-N membrane (Amersham) wetted with 10X SSC (1.5 M NaCl, 0.15 M sodium citrate) was placed on the gasket. The unused part of the membrane was covered with saran wrap. The sample template was then placed on the top. The screws were then tightened and vacuum was applied. The screws were further tightened before the vacuum was released. To all the wells to be used, 100 µl 10X SSC was added. The buffer was sucked through the membrane by vacuum. Vacuum was released when all the buffer had been passed through. RNA samples was mixed with denaturation solution to give a final concentration of 50% formamide, 6% formaldehyde and 1X MOPS buffer (0.02 M MOPS, 0.05 M sodium acetate [pH 7.0] and 0.001 M EDTA). This mixture was heated at 60°C for 10 minutes and placed on ice, 1 volume of cold 20X SSC was added to the mixture. The mixture was then applied to the membrane by vacuum. After transfer had been completed, 650 µl 10X SSC was applied for three times. The apparatus was then dismantled and the membrane was washed with 10X SSC and air dried. RNA was fixed on the membrane by UV crosslinking.

4.2.8 Preparation of radioactive labeled probes for hybridization.

4.2.8.1 Synthesis of radioactive labeled DNA probe by random labeling

By using a single LT2 bacterial colony as template, a PCR fragment was amplified using *nrdD*up2 (last chapter) and *nrdD*lpB (ACTCCATATTTGTAAGAACAGGTTATCCACAGAGG, 3' end hybridized with

position 334 of pFSH8, Fig. 3.7). The PCR condition for preparing this fragment was the same as section 4.2.5 but without the RT step. The PCR fragment was used as the template for preparing the DNA probe by Megaprime™ DNA labeling systems (Amersham). This fragment (25 ng) was mixed with 5 µl primer solution (random nonamer) in 50 µl volume and heated at 95 °C for 5 minutes, the mixture was then allowed to cool at room temperature. Ten µl labeling buffer(dATP, dGTP, dTTP in Tris-HCl [pH 7.5], 2-mercaptoethanol and $MgCl_2$), 5 µl [α-^{32}P]dCTP (3000 Ci/mmol) and 2 µl DNA polymerase I Klenow fragment (1 unit/ µl) were added to the mixture. The mixture was incubated at 37 °C for 1 hour. Five µl of 0.2 M EDTA was added to the mixture to inactivate the enzyme. The unincorporated nucleotides were removed by Nick column purification (Pharmacia). Buffer inside the Nick column was poured off and equal volume of TE buffer was added to the column and allowed to drained out. The probe was then added to the column. The column was washed with 400 µl TE and the eluent was discarded. The column was washed again with 400 µl TE buffer and the eluent was collected as probe. The probe was denatured at 95°C for 5 minutes before adding to the hybridization buffer.

4.2.8.2 Preparation of RNA probe by *in vitro* transcription.

Using pFSH8 as template, another PCR product was amplified by *nrdD*lp2 and *nrdD*up1 as described in section 4.2.6 without the RT step. This

fragment was subcloned as described in sections 3.2.5 and 3.2.6. The clone containing this fragment was designated as pNRD8, 6 μg of which was digested with 50 units of *Eco*R I in 2X one-phor-all buffer (Pharmacia) at 37°C for 1.5 hours. This enzyme digested at a site corresponding to the 5' end of the gene. The mixture was added with 20 μg proteinase K and incubated at 50°C for 30 minutes. The mixture was then extracted once with phenol : chloroform: isoamyl alcohol (25:24:1) and once with chloroform : isoamyl alcohol (24:1). The linearized plasmid was precipitated by adding 1/10 volume of 3M sodium acetate (pH 5.2) and 2 volume of 100% ethanol and kept at -20 °C for 1 hour. The DNA pellet was collected by centrifugation at 13000 rpm for 5 minutes. The DNA pellet was washed with 70% ethanol and dried and suspended in 10 μl DEPC-treated water. MAXISCRIPT™ *in vitro* transcription kit (Ambion) was used for preparation of RNA probe. One μl (about 0.5 μg) of linearized plasmid was mixed with 1X transcription buffer, 0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP, 3 μM [α-^{32}P] UTP, 10 units T3 RNA polymerase and 10 units RNase inhibitor. T3 RNA polymerase synthesizes an antisense RNA complementary to *nrdD* transcript from the phage promoter on pNRD8. The mixture was incubated at 37 °C for 1 hour. The DNA template was digested with 2 units of DNase I at 37°C for 15 minutes. Unincorporated ribonucleotides were removed by addition of 1/10 volume of 5 M ammonium acetate and 3 volumes ethanol and kept at -20 °C for 1 hour. The *in vitro*

transcript was then precipitated, washed, dried and dissolved in 100 µl DEPC-treated water. The purified *in vitro* transcript was used directly as probe.

4.2.9 Hybridization and membrane washing conditions.

For DNA blots, prehybridization and hybridization were carried out in a hybridization tube with hybridizatiion buffer (1 ml/cm^2 of membrane, 5X SSPE, 5X Denhardt's solution [0.1% BSA, 0.1% Ficoll™ and 2% PVP], 0.5% SDS and 20 µg/ml denatured sonicated salmon sperm DNA). Prehybridization was allowed to proceed at 65°C for two hours before denatured radioactive labeled probe was added. Hybridization was carried out overnight at 65°C. For RNA blots, 50% formamide was included in the hybridization buffer. Prehybridization and hybridization were carried out at 42°C for two hours and overnight respectively.

After hybridization, the membrane was washed with 2X SSPE, 0.1% SDS at room temperature for 10 minutes twice. The membrane was washed again with 1X SSPE, 0.1% SDS at 65 °C for 15 minutes twice. The membrane was finally washed with 0.1X SSPE, 0.1% SDS at 65 °C for 15 minutes.

The membrane was wrapped with Saran wrap and exposed to film (X-OMAT™, Kodak). The signal was quantified with a densitometer (Bio-Rad).

4.2.10 Normalization of samples by 16S ribosomal RNA (rRNA)

16S rRNA was chosen to normalize the sample. Normalization ensured that same quantity of RNA/DNA samples were transferred to the membrane. RT-PCR was performed as described in section 4.2.5 except primer 16SL3 (CAACCTCCAAGTAGACATCG, 3' end hybridize to the 816 bp of 16S rRNA of *S. typhimurium*) was used in 1st strand cDNA synthesis. Primer

16SU1(TGGGAAACTGCCTGATGGAG, 5' end corresponding to 124 bp of 16S rRNA of *S. typhimurium)* and primer 16SL2 (CCCACGCTTTCGCACCTGAG, 3' end hybridize to the 755 bp of 16S rRNA of *S. typhimurium*) were used in PCR amplification of cDNA (primer annealing temperature = 58 °C). RT-PCR products were sampled at every 5 cycles of amplification. The RT-PCR products were resolved on agarose and transferred to the membrane as described above. Radioactive labeled 16S rRNA specific probe was synthesized by using PCR prodcut (synthesized by using primer 16SU2 [CGCCTCACCAAGGCGACGAT, 5' end corresponding to 263 bp of 16S rRNA of *S. typhimurium*] and primer 16SL1 [CGCATTTCACCGCTACACCT, 3' end hybridize to the 680 bp of 16S rRNA of *S. typhimurium*]) as template as described in section 4.2.8.1.

4.3 Results

4.3.1 Preparation of RNA

Several distinct bands were observed when RNA was resolved by denaturing formaldehyde agarose gel (Fig. 4.1). In *S. typhimurium*, the 23S rRNA will be fragmented into several discrete bands (2.4 kb, 1.7 kb, 1.2 kb, 0.7 kb and 0.5 kb, Burgin *et al.*, 1990). Together with the 1.6 kb 16S rRNA, a series of bands with different sizes appeared on the gel. The concentrations of RNA obtained were 1 to 2 μg/μl. The OD260 to OD280 ratios were 1.4 to1.5.

4.3.2 RT-PCR of *nrdD* transcript

When using *nrdD*lp2 for the 1st strand cDNA synthesis and *nrdD*up1 plus *nrdD*lp1 as primers for PCR amplification, a 370 bp RT-PCR product was identified in both aerobic and anaerobic RNA samples (Fig. 4.2, lanes 1 and 3). Without adding reverse transcriptase in the RT reaction, this product, however, cannot be formed (Fig. 4.2, lanes 2 and 4). Therefore, the RT-PCR products were not artifacts which otherwise were generated by genomic DNA contaminations during the PCR amplification. There was no significant difference between the intensities of the RT-PCR products from aerobic and anaerobic RNA sample after 30 cycles of amplification.

When RT-PCR products from different cycles of amplification were sampled and resolved by agarose gel electrophoresis, samples from early cycles of amplification cannot be seen by ethidium bromide staining (Fig. 4.3). However, when the RT-PCR products were transferred to nylon membrane which hybridized with the

radioactive *nrdD* specific probe, the RT-PCR products was observed from the autoradiograph of the 15th cycle of amplification of the anaerobic sample, but not from that of the same cycle of amplification of aerobic sample. Moreover, differences between the intensities of RT-PCR products from aerobic and anaerobic samples were observed in 20th and 25th cycles of amplification. The autoradiograph with the hybridization signals was shown in Fig 4.4

The relative levels of signals were quantified by a densitometer and expressed as adjusted volume OD X mm^2. The ratios of the relative levels of signal of anaerobic sample to that of aerobic sample were: 24-fold at the 20th cycles, 6-fold at the 25th cycle and 1.5-fold at the 30th cycles. There were no difference in relatvie levels of signal when using 16S rRNA primers for RT-PCR on the same RNA samples and 16S rRNA specific probe for quantitation (Fig. 4.5).

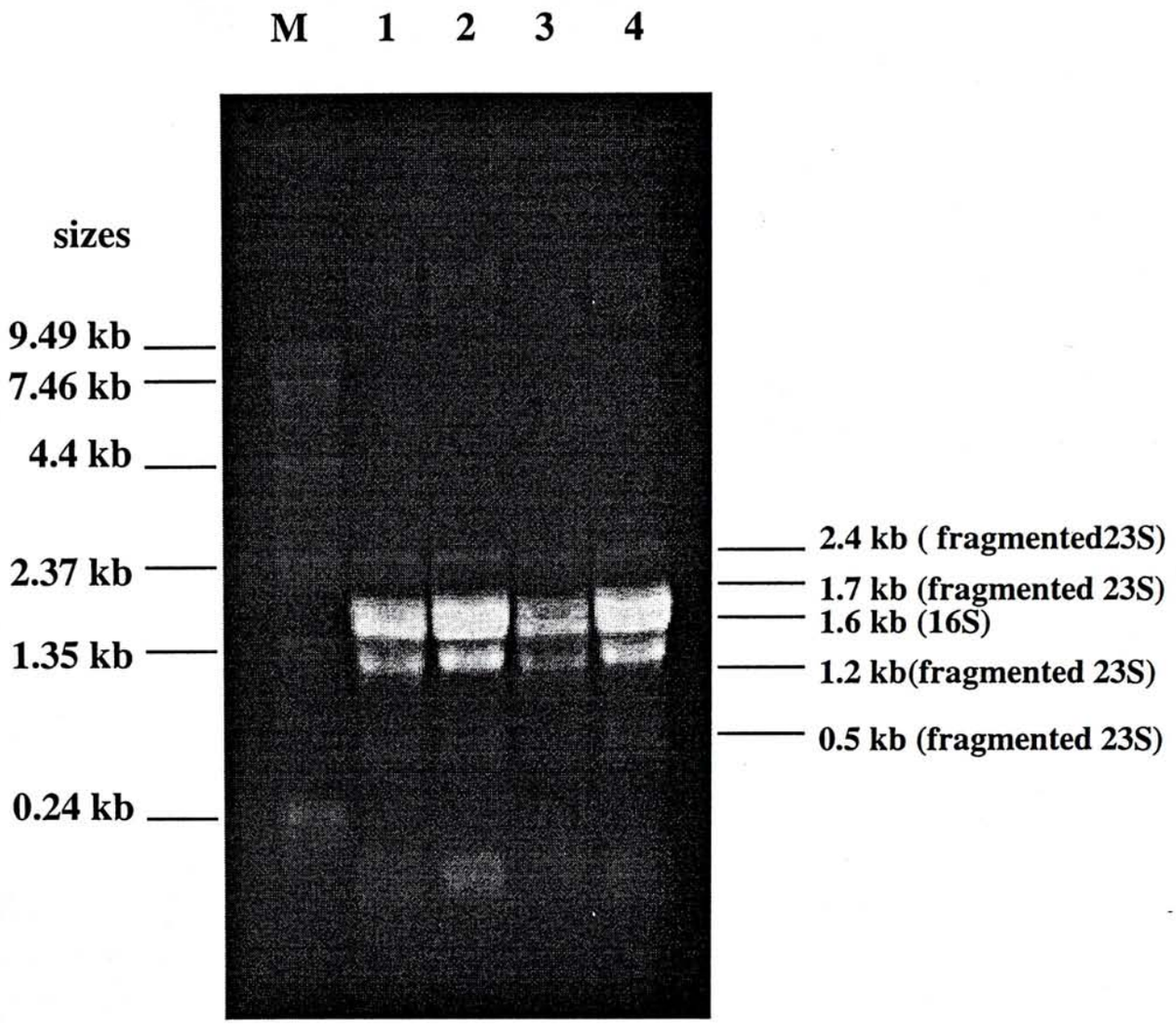

Fig. 4.1 Total RNA prepared from *S. typhimurium* in aerobic and anaerobic environemnts resolved by 1.5% denaturing formaldehyde agarose gel electrophoresis. Lane M: RNA marker (Gibco BRL). Lane 1: 5 μg of total aerobic RNA. Lane 2: 10 μg of total aerobic RNA . Lane 3: 5 μg of total anaerobic RNA. Lane 4: 10 μg of total anaerobic RNA. Note that the 23S rRNA of *S. typhimurium* is fragemented as indicated.

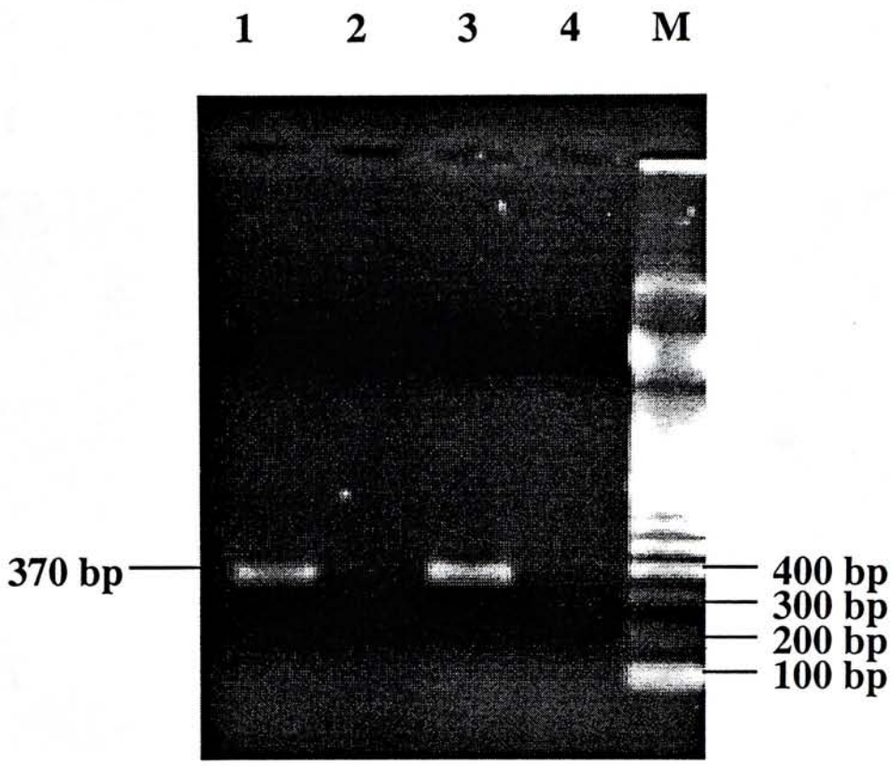

Fig. 4.2 RT-PCR of *nrdD* transcript after 30 cycles of amplification resolved by agarose gel electrophoresis. A 370 bp RT-PCR product was formed by using nrdDlp2 in 1st strand cDNA synthesis and nrdDlp1 plus nrdDup1 in PCR amplification of cDNA template. Lane 1: RT-PCR product formed from aerobic RNA sample with Reverse transciptase in RT. Lane 2: same as lane 1 but without reverse transcriptase added in RT. Lane 3: RT-PCR product formed from anaerobic RNA sample with Reverse transciptase in RT. Lane 4: same as lane 3 but without reverse transcriptase added in RT. Lane M: 100 bp marker (Pharmacia).

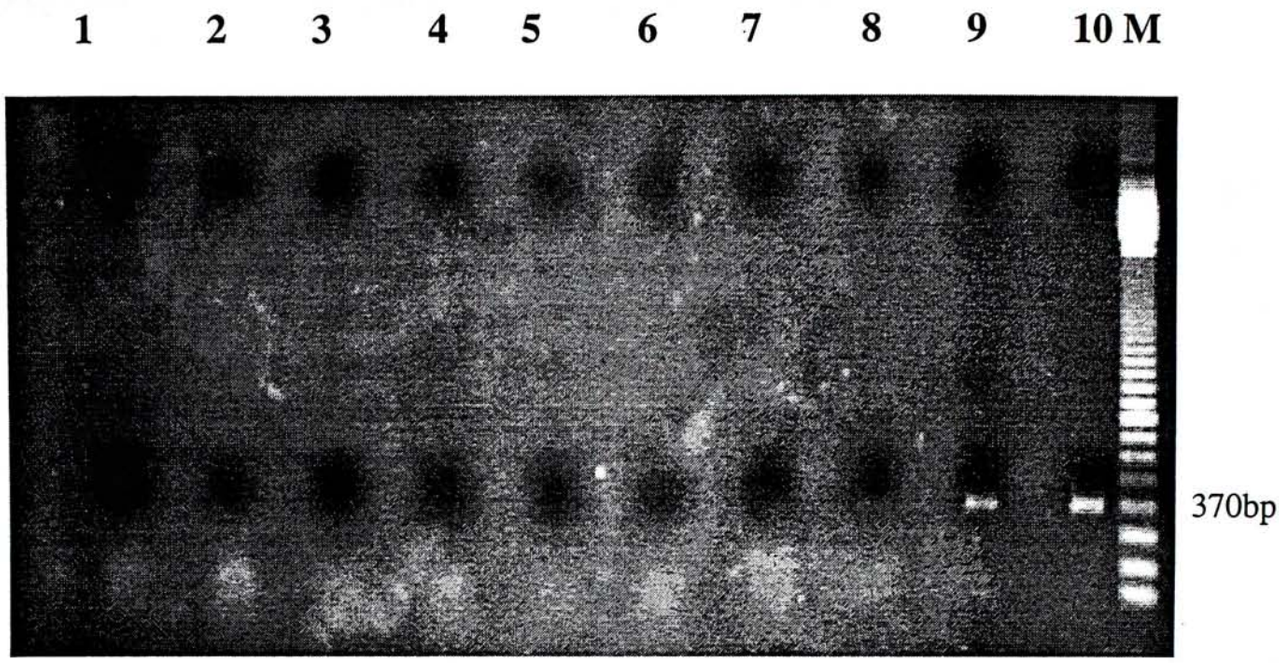

Fig. 4.3 RT-PCR of *nrdD* transcript under different cycles of amplification. Conditions are the same as in Fig. 4.2. Lane 1: RT-PCR product of aerobic RNA at 10th cycle of amplification. Lane2: RT-PCR product of anaerobic RNA at 10th cycle of amplification. Lane3: RT-PCR product of aerobic RNA at 15th cycle of amplification. Lane 4: RT-PCR product of anaerobic RNA at 15th cycle of amplification.Lane 5: RT-PCR product of aerobic RNA at 20th cycle of amplification. Lane 6: RT-PCR product of anaerobic RNA at 20th cycle of amplification. Lane 7: RT-PCR product of aerobic RNA at 25th cycle of amplification. Lane 8: RT-PCR product of anaerobic RNA at 25th cycle of amplification. Lane 9: RT-PCR product of aerobic RNA at 30th cycle of amplification. Lane 10: RT-PCR product of anaerobic RNA at 30th cycle of amplification. Lane M: 100 bp marker (Pharmacia)

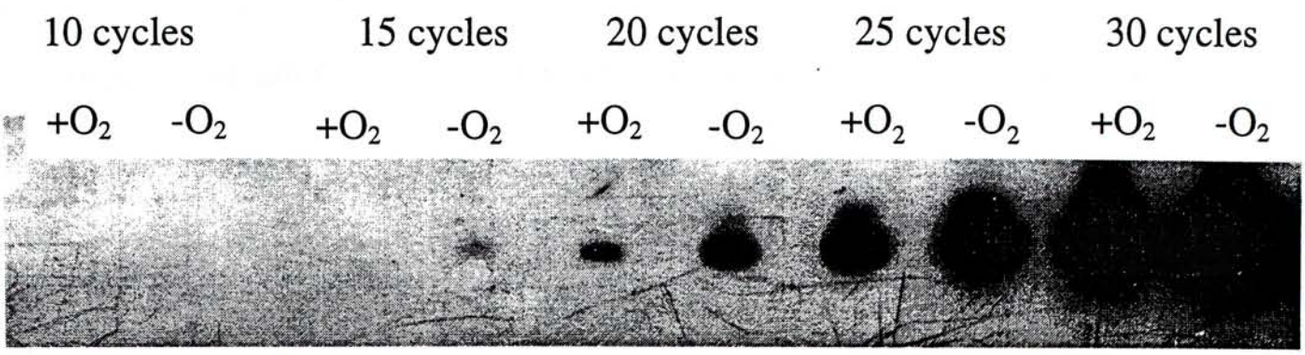

Fig. 4.4 Autoradiograph showing RT-PCR products amplified with *nrdD* specific primers hybridized with *nrdD* specific probes. The arrangement is same as fig. 4.5.

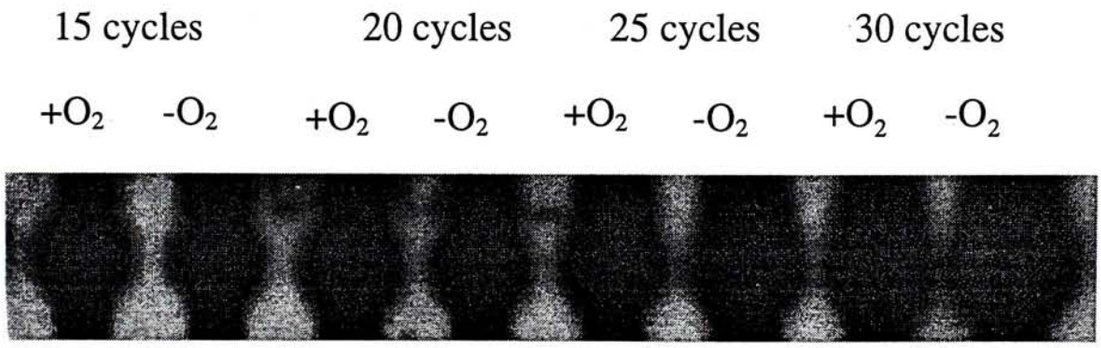

Fig. 4.5 Autoradiograph showing RT-PCRproducts amplified with 16S rRNA specific primers (using same RNA samples as fig 4.4) hybridized with 16S rRNA specific probes.

4.3.3 Northern blot analysis of *nrdD* transcript

In Northern blot analysis, two different amounts of total RNA, 5 μg and 10 μg, were loaded into the gel. A 2.4 kb transcript can be identified in both 5 μg and 10 μg anaerobic RNA samples. The signal in the 10 μg sample was slightly stronger than the 5 μg sample. However, this signal cannot be identified in both 5 μg and 10 μg aerobic RNA sample. Moreover, the *nrdD* specific antisense RNA probe gave strong background signals in positions corresponding to rRNA even under several stringent washes. The background signals were the same between aerobic and anaerobic samples of the same amount. The autoradiograph with the hybridization signals was shown in Fig. 4.6.

4.3.4 RNA dot blot analysis of *nrdD* expression in an *oxrA* mutant.

oxrA::Tn10 mutation was transduced form parent TN2336 to recipient LT2 by P22 transduction. Transductants grown on LB plate with tetracycline were characterized on MacConkey-nitrate plate anaerobically. Wild type LT2 appeared as large white colonies, while TN2336 and transductants appeared as tiny deep red colonies. The transductant having *oxrA*$^-$ phenotype was designated as WL9601. The expression levels of *nrdD* transcripts in LT2 and WL9601 were compared with RNA dot blot analysis.

Total RNA (each of 5 μg and 10 μg) from LT2 and WL9601 was transferred onto a nylon membrane with a dot-blot apparatus, the intensities of the hybridization signals with the *nrdD* specific probe were compared with each other. The autoradiograph with the hybridization signals was shown as Fig. 4.7. All signals from

5 μg RNA were weaker than that from 10 μg RNA in both aerobic and anaerobic RNA samples. For wild type LT2, with the same amount of RNA, the signals from anaerobic RNA samples were stronger than that from aerobic RNA samples. The signals from aerobic RNA samples of WL9601, both 5 μg and 10 μg, were the same as those from wild type. However, the signals from anaerobic RNA samples, both 5 μg and 10 μg, of WL9601 were weaker than those from wild type LT2. The signals from *oxrA* mutant anaerobic RNA samples were even weaker than those of wild type aerobic RNA samples. The differences in hybridization signals were not found when using 16S rRNA specific probe hybridized with the same RNA samples as shown in Fig. 4.8.

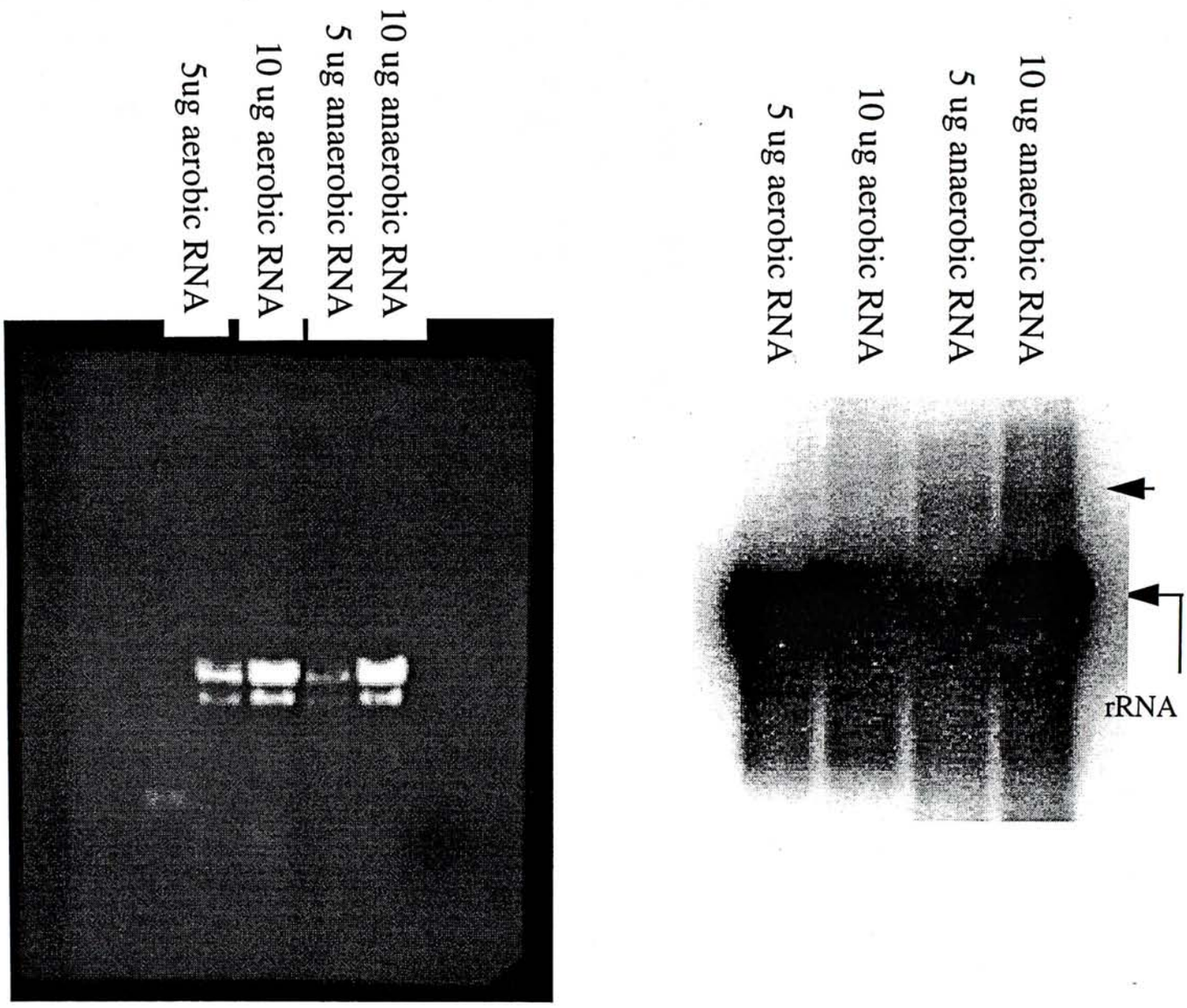

Fig. 4.6 Panel A: Filter showing ethidium bromide stained RNA after Northern blotting. Panel B; Autoradiograph showing RNA hybridized with antisense *nrdD* specific RNA probe. Arrow shows the position of *nrdD* transcript (2.4 kb)

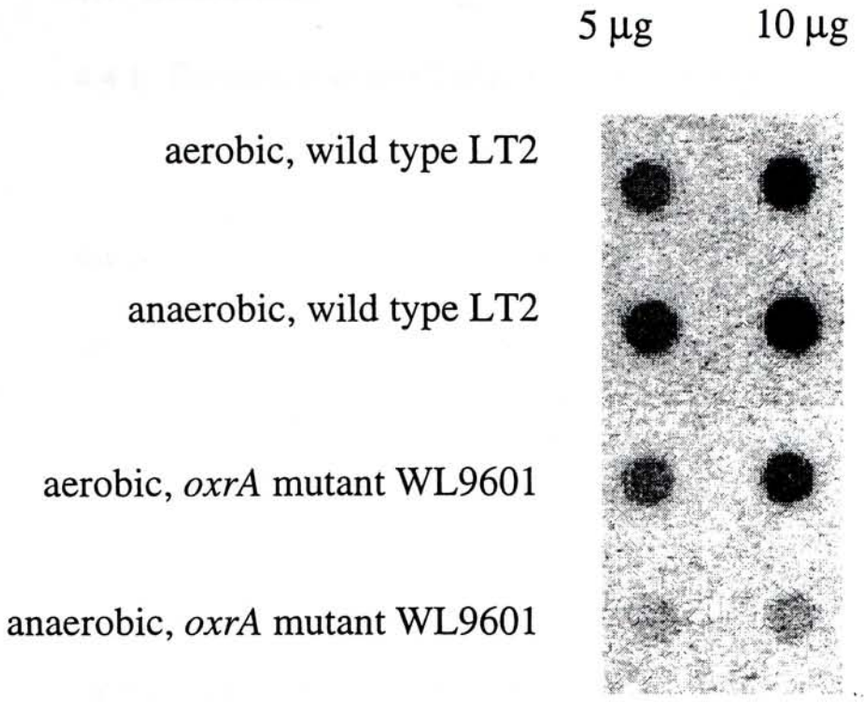

Fig. 4.7Autoradiograph showing different samples of RNA hybridized with *nrdD* specific probe.

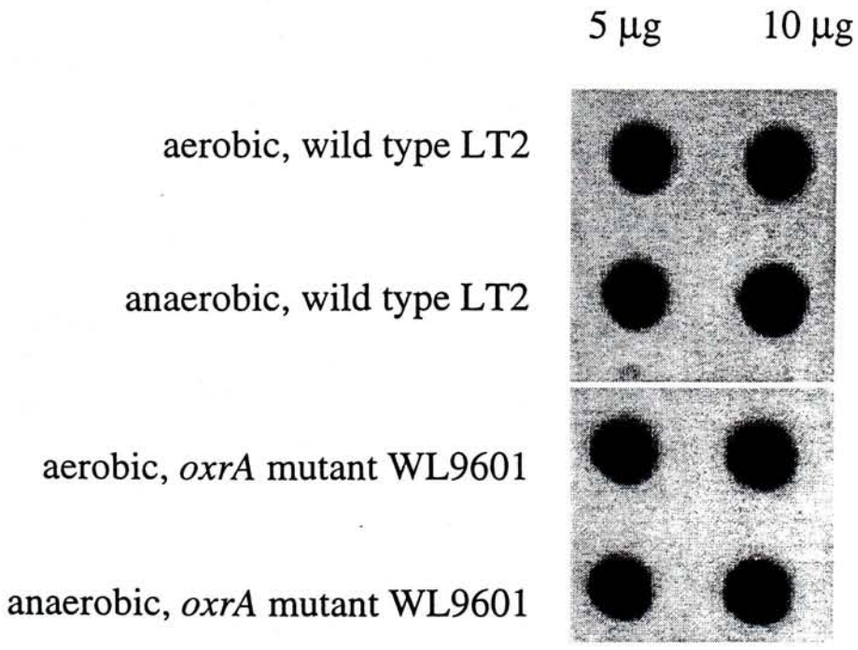

Fig. 4.8 Autoradiograph showing different samples of RNA hybridized with 16S rRNA specific probe.

4.4 Discussions

4.4.1 Expression of *nrdD* of *S. typhimurium* in aerobic and anaerobic environments.

Different approaches were used to investigate the expression levels of *nrdD* transcripts of *S. typhimurium* in different environments. By RT-PCR, the expression level of *nrdD* transcripts was found to be up-regulated during anaerobiosis. The difference was between 24-fold to 1.5-fold depending on number of cycles amplified. The difference decreased when number of cycles amplified increased. Theoretically, PCR is an exponential amplification of a DNA template while the extent of amplification (Y) is governed by initial amount of template (A), efficiency of amplification (R) and number of cycles (n) by formula $Y = A(1+R)^n$ (Chelly and Kahn, 1996). However, the exponential increase of the extent (Y) with a constant efficiency (R) only happened in early cycles of amplification (Chelly and Kahn, 1996) because of limiting factors present in the amplification reaction. Hence, the amount of transcripts in aerobic and anaerobic samples should be compared when they were still exponentially amplified in early cycles. Moreover, in order to compare the differences in the amounts of the transcript, it should be assumed that the initial amount of the *nrdD* mRNA is proportional to the amount of the cDNA being reverse transcribed. This assumption can only be fulfilled by using the same primers, the same reverse transcriptase and the same conditions for reverse transcription and amplification. However, a small difference in the reaction will drastically affect the amount of the amplification products. Therefore, an earlier cycle will give a more accurate result. So, the difference between transcript of *nrdD* in *S. typhimurium* in aerobic and anaerobic environments was at least 24 fold which was determined in the

20th cycles of amplification. It has been suggested that minor mRNA ceases to be exponentially amplified after 20 cycles (Chelly and Kahn, 1996). Therefore, comparison of *nrdD* transcripts between aerobic and anaerobic RNA samples at the 20th cycles is appropiate.

Northern blot hybridization analysis gave similar results. The *nrdD* transcripts expressed at a higher level in anaerobic environment than aerobic environments. Moreover, the size of the transcript was determined to be approximately 2.4 kb. The ORFs of *nrdD* and *nrdG* are about 2140 bp and 460 bp respectively, the intervening sequence is about 120 bp, if the total size of the 5' and 3' untranslated sequences are assumed to be 300 bp, the primary length of *nrdDG* transcript is about 3 kb if there is no post-transcriptional processing. Since the probe was specific to *nrdD* only, the transcript detected would be corresponding to the length of *nrdD*. Therefore, the primary transcript may be processed after transcription to give the 2.4 kb *nrdD* transcript.

It should be noted that the transcript cannot be found in the aerobic sample by Northern blot analysis. However, the transcript can be found in aerobic sample by using RT-PCR. Since RT-PCR is a more sensitive method than Northern blot analysis, the *nrdD* transcript probably present in the aerobic RNA sample but at a level too low for Northern blot analysis to detect. There is evidence of aerobic expression of *nrdD*. In aerobically grown *E. coli*, small anaerobic ribonucleotide reductase activity can be detected (Garriga *et al.*, 1996) indicating that the transcript is also expressed aerobically.

The effect of *oxrA* mutation on *nrdD* expression was investigated by RNA dot blot analysis. The expression level of *nrdD* transcripts was found to be decreased in an *oxrA* mutant anaerobically. Moreveor, the basal expression of *nrdD* in aerobic environment did not depend on Fnr (encoded by *oxrA*) since the signals generated by the wild type and the *oxrA* mutant were the same. So, there seem to be no substantial aerobic control of *oxrA* on *nrdD* expression. Thus, Fnr act as anaerobic transcriptional activator on *nrdD* expression. Fnr may bind to the putative binding sites on the promoter of *nrdD*, interacts with RNA polymerase and increases the expression of *nrdD*. It is rather extraordinary that the level of expression of *nrdD* of *oxrA* mutant in anaerobic environments was lower than that in aerobic environment. It may be due to involvement of unknown anaerobic repressor. The expression of this repressor may be repressed by Fnr anaerobically. In the *oxrA* mutant, this repressor may be derepressed since Fnr is absent. Hence, this repressor may be present in extraordinary amount and strongly represses *nrdD* transcription. The other possibility is that certain metabolites can act as an effector molecule of *nrdD* transcription. If production of this effector molecule is under Fnr regulation, loss of Fnr would result in imbalance of this effector molecules and *nrdD* expression would also be affected.

The biochemical properties of NrdD is similar to pyruvate formate lyase (Pfl). Both of them carry a glycyl radical important for catalysis. The glycyl radicals of both enzymes are oxygen sensitive. Both enzymes will be truncated at this glycyl radical site when they are exposed to oxygen (King and Reichard, 1995; Sun *et al*, 1993; Wagner *et al.*, 1992). Pfl expression increased in anaerobiosis in *E. coli* and *S.*

typhimurium (Sawers and Bock, 1988; Wong *et al.*, 1989). The regulation of Pfl depended on Fnr (Kaiser and Sawers, 1995 and 1997; Sawers, 1993; Sawers and Bock, 1988 and 1989; Sawers and Suppmann, 1992; Wong *et al.*, 1989). This regulation would probably prevent wastage to the bacterial cell so that unwanted or useless gene products would not be synthesized. The same phenomenon was observed in aerobic ribonucleotide reductase (NrdAB) of *E .coli*, the expression of this enzyme was decreased in anaerobic condition (Casado *et al.*, 1991). Therefore , NrdD and Pfl seem to make use of the same regulatory mechanism to prevent this wastage at transcriptional level with Fnr as the common activator.. Thus, the similarity of NrdD to Pfl extend from biochemical properties to genetic regulation. The similarity can be arisen from either divergent evolution of a common ancestor protein or convergent evolution of two distant proteins adapting the anaerobic environment. To clarify this problem, sturctural properties of these two enzymes should be investigated.

4.4.2 Experimental design

In this study, I had measured the *nrdD* transcript by different approaches. By RT-PCR, the transcript in aerobic environment can be detected due to its high sensitivity. However, this approach cannot give the size of the transcript. On the other hand, Northern blot analysis can determine the size of the transcript but the sensitivity is lower than that of RT-PCR. Combination of both can compensate the drawback of each other.

Only the effect of oxygen availability had been investigated in this study, however, many genes involved in anaerobic metabolism are regulated by different

electron acceptor such as nitrate, furmurate and TMAO as well as catabolite repression. The effect of these regulator need to be characterized in further studies.

The probe employed in this study is specific to *nrdD* only, by nucleotide sequence analysis in last chapter, no promoter sequence can be detected in between *nrdD* and *nrdG*, therefore, these two genes are organized in an operon. Whether the expression of these two genes are coordinated needs further characterization using *nrdDG* and *nrdG* specific probes.

16S rRNA specifc probe were employed in the experiment to normalized the RNA samples. The 16S rRNA specific probe should hybridize to the RNA samples with equal intensities if same amounts of RNA are present. The intensities of the signals should not depend on the oxygen availiability. It ensured that the differences between the signals when using *nrdD* specific probe were due to the abundance of the mRNA investigated, but not due to the differences of amount of RNA transferred to the membrane.

In the Northern blotting experiment, rRNA cross hybridized with *nrdD* specific probe. It may be due to there is sequence homology between rRNA and the probe used. The cross hybridization cannot be taken into account in the dot blot analysis since the probes and the hybridization conditions employed in both experiments were different.

Chapter 5

Characterization of *nrdD*::Tn*10* mutant of *S. typhimurium*

5.1 Introduction

In *E. coli*, three different ribonucleotide reductases are present (Reichard, 1997). The first one is the aerobic ribonucleotide reductase (NrdAB) which functions in aerobic environment. The second one is the anaerobic ribonucleotide reductase (NrdDG) which functions in anaerobic environments. The last one is a cryptic ribonucleotide reductase (NrdEF). The function of NrdEF is not known but the homologues of this enzyme is functional in other species (Jordan *et al.*, 1996b). The genes encoding these three different enzymes are also identified in *S. typhimurium* (Jordan *et al.*, 1994a, 1994b, 1995; this study). The presence of three functionally similar enzymes in one genome raises an interesting question: can these enzymes complement each other? If they can complement each other, loss of one enzyme would not affect growth.

A Tn*10* insertion mutant which cannot rescue a temperature sensitive (ts) *nrdA* mutation anaerobically was isolated. (J. Roth, personal communication). This Tn*10* mutation was designated as *zzz*-3875::Tn*10*. This insertion mutation was proposed to be located in *nrdD* but had not yet be confirmed since no genetic markers are available that neither conjugational nor transductional analysis can be used to locate the position of the mutation. However, the location of mutation can be determined by Southern blotting analysis using *nrdD* specific probes,. In this chapter, *zzz*-3875::Tn*10* was confirmed to be *nrdD*::Tn*10* mutation and characterized

5.2 Materials and methods

5.2.1 Bacteria and bacteriophages strains.

TT14888(dna-98 [*nrdA*$_{ts}$] *zzz*-3875::Tn*10*) and TT14889 (dna-98 [*nrdA*$_{ts}$] *zzz*-3876::Tn*10*) are *S. typhimurium* LT2 derived strains (J. Roth, personal communication). P22 HT *int* was used in transduction of *S. typhimurium*.

5.2.2 Transduction of *zzz*-3875::Tn*10* to *S. typhimurium*.

Insertion mutation *zzz*-3875::Tn*10* was transduced to LT2 by P22 transduction as described in section 4.2.3.2. The transductant isolated on LB plate with 15 μg/ml tetracycline was designated as WL9701.

5.2.3 Characterization of *zzz*-3875::Tn*10* by Southern hybridization.

5.2.3.1 Preparation of genomic DNA from *S. typhimurium*.

Genomic DNA was prepared from LT2, TT14888 and TT14889 as described (Owen and Borman, 1987). Overnight cultures (1.5 ml each) of LT2, TT14888 and TT14889 were collected by centrifugation at 13000 rpm for 5 minutes. The bacterial pellet was washed with 500 μl SE buffer (150 mM NaCl and 100 mM EDTA [pH 8.0]). The suspension was centrifuged at 13000 rpm for 5 minutes. The bacterial pellet was suspended in 480 μl SET buffer (150 mM NaCl, 15 mM EDTA [pH 8.0] and 60 mM Tris-HCl [pH 8.3]), and added with 50 μl 10% SDS, 1.25 μl proteinase K (20 mg/ml) and 2.5 μl RNase A (10 mg/ml). The mixture was incubated at 50°C for 20 minutes. After incubation, the mixture was extracted with phenol : chloroform : isoamyl alcohol (25:24:1) for three times. The aqueous layer was then extracted with chloroform : isoamyl alcohol (24:1) twice. DNA was precipitated from the

aqueous layer by adding 1/10 volume of 3M sodium acetate (pH 5.2) with 2 volume of 100% ethanol and kept at -20 oC for 1 hour. The genomic DNA was collected by centrifugation at 13000 rpm for 15 minutes at 4 °C. The DNA pellet was washed with 70% ethanol and dried under vacuum. DNA was dissolved in 100 µl sterile ultrapure water. The quality of the genomic DNA was analyzed by 1% agarose gel electrophoresis in 1X TBE buffer. The quantity of the genomic DNA was measured by UV spectrophotometry at 260 and 280nm using GeneQuant machine (Pharmacia).

5.2.3.2 Restriction enzyme digestion of genomic DNA and Southern hybridization

Genomic DNA of LT2, TT14888 and TT14889 (each 2 µg) was digested with 40 units of *Eco*R I or *Pvu* I restriction enzyme in 2X One-Phor-All Buffer Plus (20 mM Tris-acetate [pH 7.5], 20 mM Magnesium acetate and 100 mM potassium acetate) in a 50 µl reaction volume. The reaction mixture was incubated overnight at 37°C and the enzyme was inactivated at 85°C for 30 minutes. The restriction fragments were resolved by 1% agarose gel electrophoresis in 1X TBE. Resolved restriction fragments were transferred to Hybond-N membrane (Amersham) as described in section 4.2.7.1. The radioactive labeled *nrdD* specific probe was prepared by random labeling as described in section 4.2.8.1. Inserts from pNWL8 and pNWL11 (chapter 3), which carry *S. typhimurium nrdD* structural gene, were used as template for probe synthesis. Hybridization, membrane washing and autoradiography were performed as described in section 4.2.9.

5.2.4 Characterization of growth pattern of *nrdD*::Tn*10* mutant.

Anaerobic incubation of liquid culture was achieved by filling test tubes (Bellco) with LB medium (with or without tetracycline, 15 μg/ml) to the top (about 20 ml) and the tubes were stoppered after inoculation. Growth was started by 1% inoculation with overnight culture grown in the LB medium aerobically at 37°C. Aerobic growth of liquid culture was achieved by growing 50 ml culture in a 250 ml conical flask shaking at 200 rpm. Three incubation temperatures, 30, 37 and 42°C, were tested. The absorbance of the culture was measured at 600nm by a spectrophotometer. Aerobic culture was sampled directly from the flask. Anaerobic sample was drawn from the test tube by purging a 1-ml syringe through the stopper.

5.3 Results

5.3.1 Characterization of *zzz*-3875::Tn*10* in *S. typhimurium*.

The location of Tn*10* insertions in TT14888 and TT14889 were determined by Southern blotting analysis. Firstly, the genomic DNA was extracted from wild type LT2, TT14888 and TT14889 and resolved with agarose gel electrophoresis (Fig 5.1). The concentration of the genomic DNA was about 1 μg/μl. Genomic DNA from different strains was cut by restriction enzyme *Eco* RI or *Pvu* I separately. The *Eco*R I or *Pvu* I digested genomic DNA from different strains resolved by agarose gel electrophoresis were shown in Fig.5.2. The sizes of the restriction fragments ranged from 0.5 kb to 15 kb. After transferring the restriction fragments to a solid support, a radioactive-labeled *nrdD* specific probe was hybridized with the immobilized DNA. Autoradiograph with hybridization signals was shown in Fig. 5.3. From the genomic DNA of LT2 digested with *Eco* RI, a hybridization signal of approximately 6.5 kb was found. From the genomic DNA of TT14888 digested with *Eco* RI, two hybridization signals corresponding to 7 kb and 9 kb were identified. The hybridization signals of TT14889 *Eco* RI digested genomic DNA were identical to those of TT14888. Three hybridization signals were identified in *Pvu* I digested LT2 genomic DNA from the autoradiograph,, two of them were about 2 kb (appeared as a single band) and the other one was 6 kb. From the *Pvu* I digested TT14888 genomic DNA, three hybridization signals corresponding to 2 kb, 6 kb and 11 kb were identified. The same restriction pattern was observed in TT14889 *Pvu* I digested genomic DNA. The restriction fragment length polymorphisms shown between LT2 and TT14888/TT14889 revealed that the insertions of Tn*10* were inside the *nrdD*

structural gene. Thus, the genotype of TT14888 or TT14889 should be dna-98 [$nrdA_{ts}$] *nrdD*::Tn*10*. After confirming *zzz*-3875::Tn*10* was located inside *nrdD*, this mutation from TT14888 was moved to wild type LT2 by P22 transduction, the tetracycline resistant transductant obtained was WL9701 with genotype $nrdA^{+}$ *nrdD*::Tn*10*.

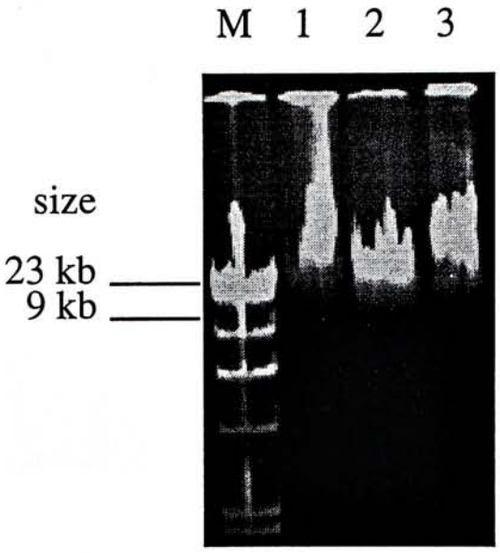

Fig. 5.1 Genomic DNA from different *S. typhimurium* strains resolved by 1% agarose gel electrophoresis. Lane M: λ *Hin* dIII marker. Lane 1: genomic DNA from LT2. Lane 2: genomic DNA from TT14888. Lane 3: genomic DNA from TT14889.

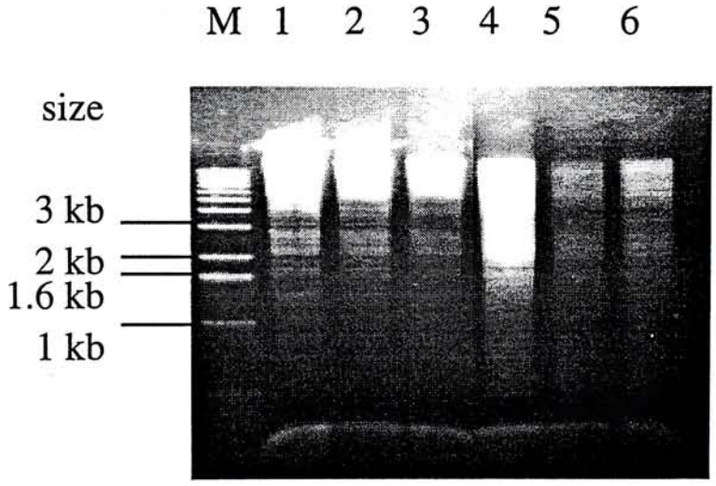

Fig. 5.2 Genomic DNA of different strains cut by *Eco* RI and *Pvu* I resolved by agarose gel electrophoresis. Lane M: 1-kb ladder (Gibco BRL). Lane 1: LT2 genomic DNA cut by *Eco* RI. Lane 2: TT14888 genomic DNA cut by*Eco* RI. Lane3: TT14889 genomic DNA cut by *Eco* RI. Lane 4: LT2 genomic DNA cut by *Pvu* I. Lane 5: TT14888 genomic DNA cut by *Pvu* RI. Lane6: TT14889 genomic DNA cut by *Pvu* RI.

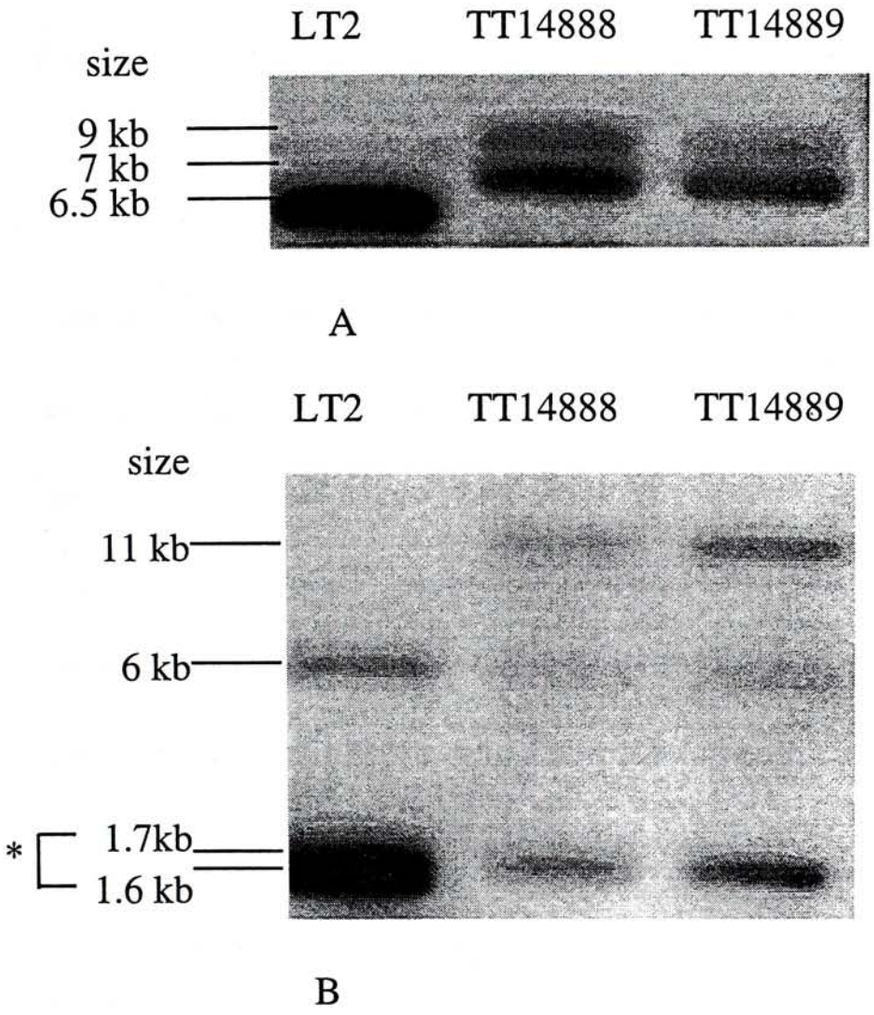

Fig.5.3 Autoradiograph showing restriction fragment length. polymorphisms(RFLP) between LT2, TT14888 and TT14889. Panel A: RFLP of genomic DNA cut by *Eco* RI. Panel : RFLP of genomic DNA cut by *Pvu* I.
*appeared as single band in the autoradiograph.

5.3.2 Characterization of growth pattern of *nrdD* mutant.

The growth patterns of LT2, TT14888 and WL9701 were determined in aerobic and anaerobic environments at 30, 37 and 42°C separately. Cell density (OD600) was plotted against time of growth as shown in Fig.5.4a-f. The growth characteristics of different strains at different culture conditions were listed in Table 5.1. The doubling times were always shorter in aerobic cultures than those of anaerobic cultures of all strains. Moreover, the increases in biomass, which represented by change in OD600, were always higher in aerobic cultures than those of anaerobic culture of all strains. The doubling times in aerobic cultures at all temperatures for all strains were 30-40 minutes. The doubling times in anaerobic culture at 37 and 42°C were 40-50 minutes, while the doubling times of anaerobic culture at 30°C were 60 and 70 minutes for LT2 and TT14888 respectively. The doubling times of different strains in the same culture condition did not show a great difference. Except in two situations, the maximum OD600 at stationary phase of different strains in the same culture condition was roughly the same. The first difference occurred in aerobic culture of TT14888 at 42°C. While the other two strains can grow to maximum OD600 of about 2.6, TT14888 can only grow to maximum OD600 of about 0.9. The efficiency of increase in biomass (change in OD600) of TT14888 was only 32% in this growth condition when compared to the wild type LT2. The other difference was found in the anaerobic culture of TT14888 at 42°C. While the other two strains can grow to a maximum OD600 of about 0.35, TT14888 can only grow to a maximum OD600 of 0.23. The loss in biomass increase (change in OD600) of this strain in this culture condition was 50% (0.32 Vs 0.16).

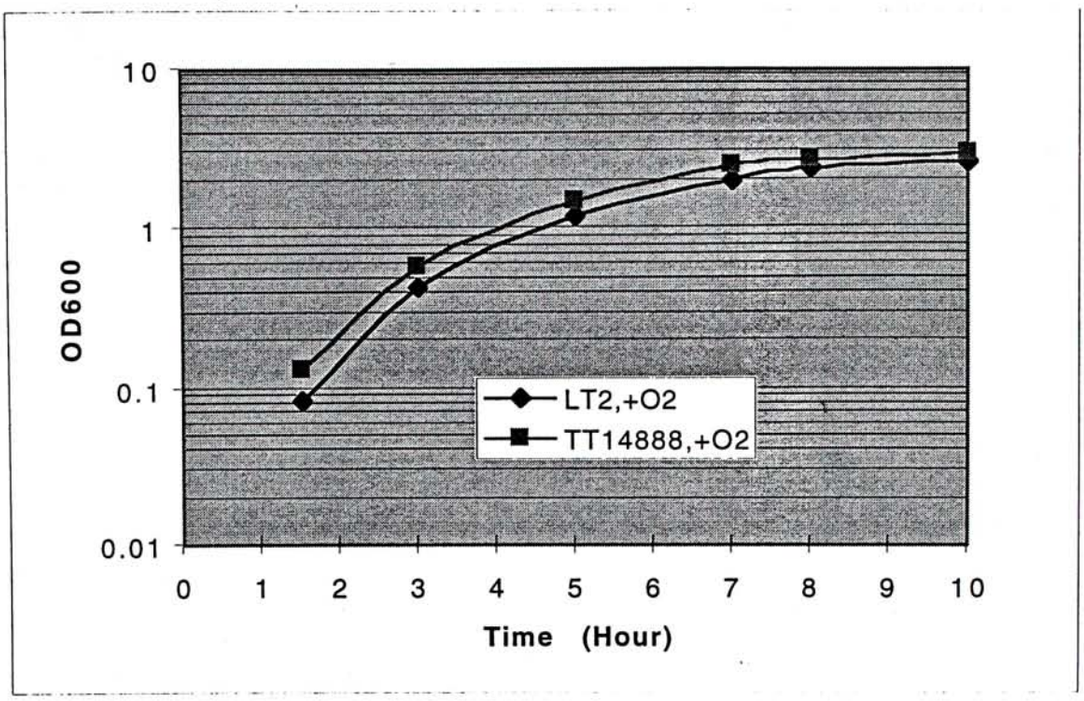

Fig. 5.4a Growth curve showing aerobic growth of LT2 and TT14888 at 30 °C.

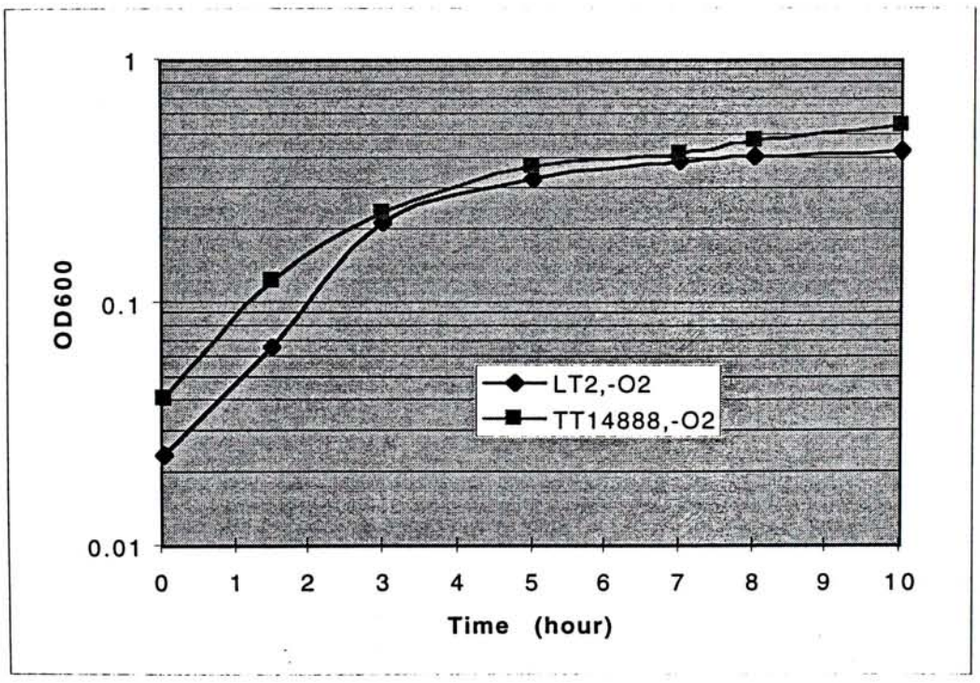

Fig. 5.4b Growth curve showing anaerobic growth of LT2 and TT14888 at 30 °C.

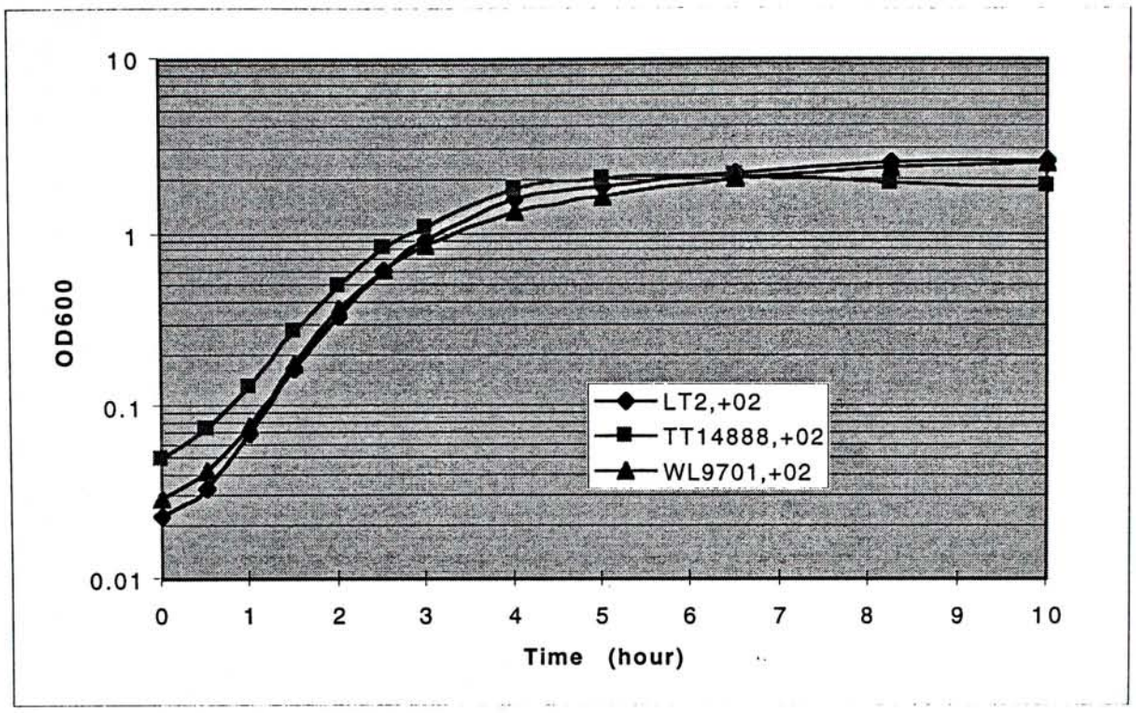

Fig. 5.4c Growth curve showing aerobic growth of LT2, TT14888 and WL9701 at 37 °C.

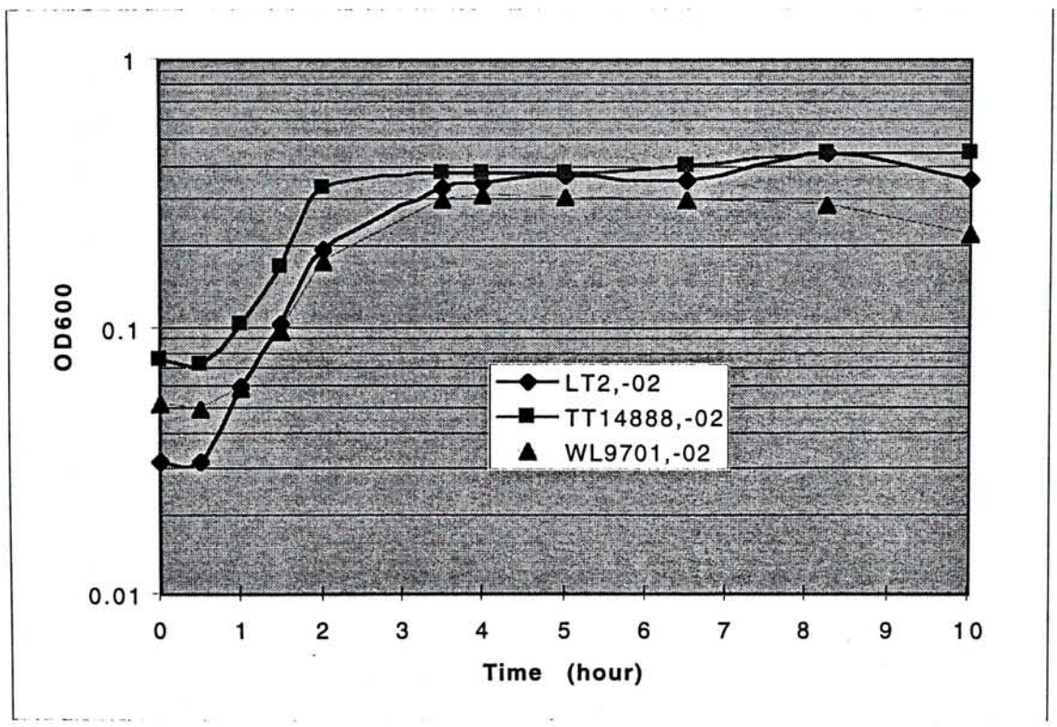

Fig. 5.4d Growth curve showing anaerobic growth of LT2, TT14888 and WL9701 at 37 °C.

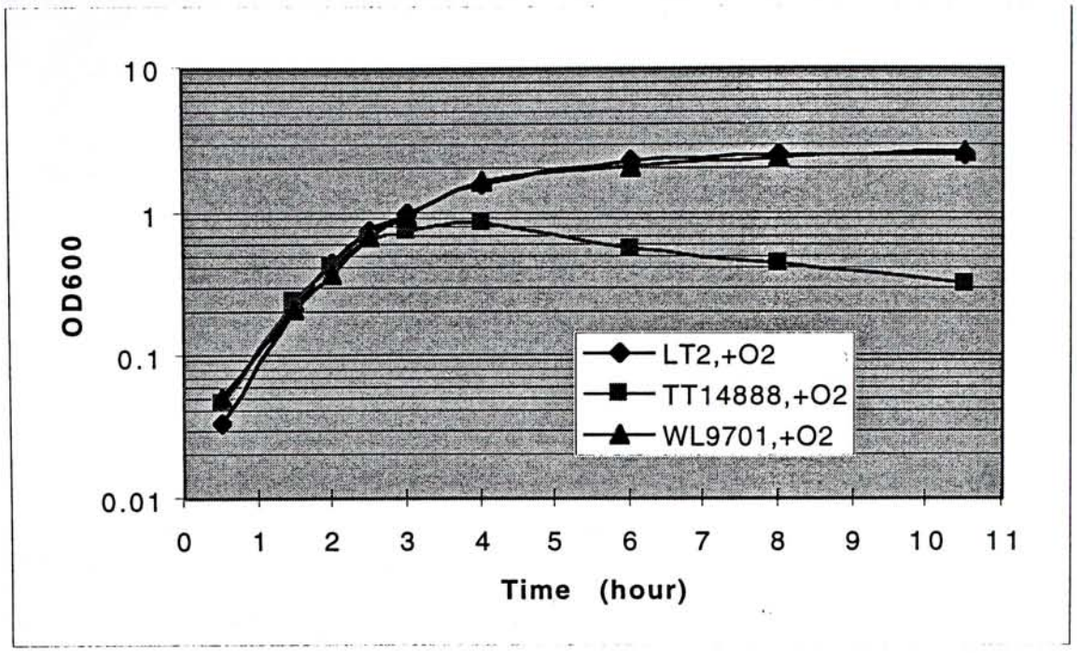

Fig. 5.4e Growth curve showing aerobic growth of LT2, TT14888 and WL9701 at 42 °C.

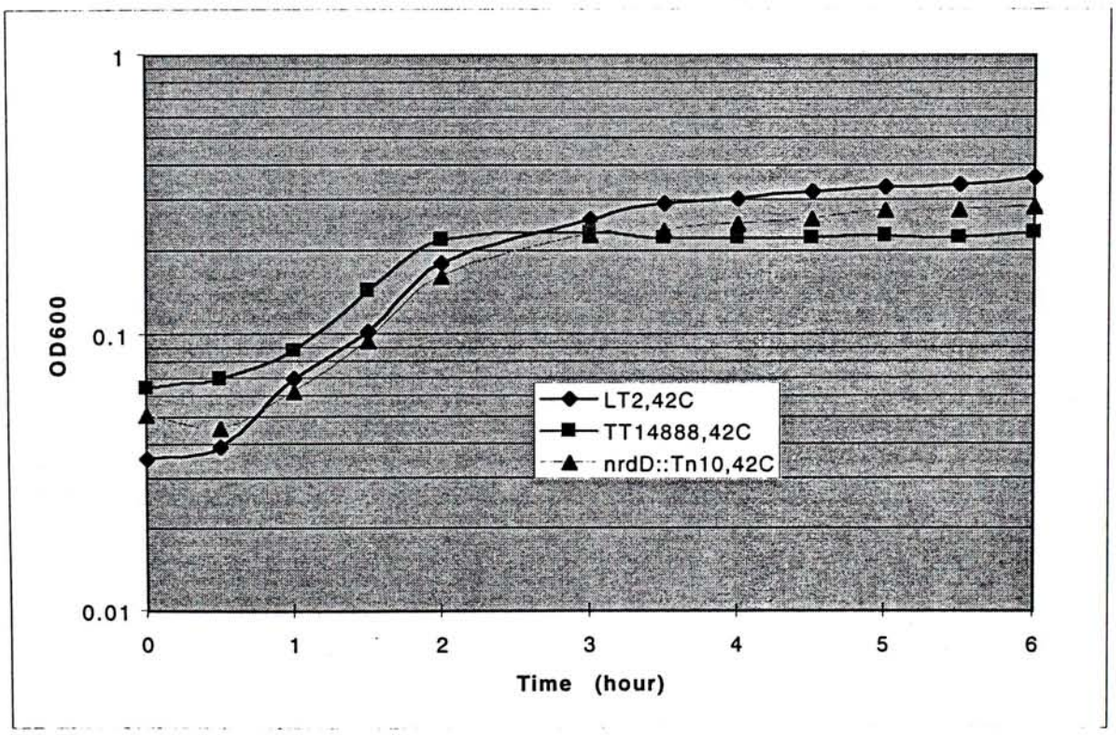

Fig. 5.4f Growth curve showing anaerobic growth of LT2, TT14888 and WL9701 at 42 °C.

Table 5.1 Characteristics of growth of LT2, TT14888 and WL9701 in different culture conditions

culture conditions	strains	doubling time at exponential phase (mine)	maximum OD600 at stationary phase	change in OD600[#] (efficiency)[♦]
aerobic, 30°C	LT2	36	2.6	2.55 (100%)
	TT14888	39	2.9	2.88 (112%)
	WL9701	ND[*]	ND	ND
anaerobic, 30°C	LT2	60	0.42	0.40 (100%)
	TT14888	69	0.54	0.50 (125%)
	WL9701	ND	ND	ND
aerobic, 37°C	LT2	28	2.58	2.56 (100%)
	TT14888	37	2.17	2.12 (83%)
	WL9701	32	2.56	2.53 (99%)
anaerobic, 37°C	LT2	39	0.46	0.43 (100%)
	TT14888	39	0.45	0.37 (86%)
	WL9701	42	0.32	0.27 (63%)
aerobic, 42°C	LT2	28	2.57	2.54 (100%)
	TT14888	30	0.87	0.82 (32%)
	WL9701	30	2.66	2.61 (97%)
anaerobic, 42°C	LT2	43	0.36	0.32 (100%)
	TT14888	50	0.23	0.16 (50%)
	WL9701	45	0.34	0.29 (91%)

#change in OD600 was obtained by subtracting maximum OD600 with initial OD600
*not determined
♦efficiency was compared with wild type LT2 which was defaulted as 100%

5.4 Discussions

The position of *zzz*-3875::Tn*10* of TT14888 cannot be located in the *S. typhimurium* genome by traditional conjugation or transduction mapping methods, since there was no suitable genetic marker for selection. The map position of *nrdD* was about 96 minutes in *S. typhimurium* genome (Wong, personal communication). There was no selectable auxotrophic markers in this region (Sanderson *et al.*, 1995), therefore, mapping of this insertion mutation became impossible by these approaches. However, this insertion can be characterizd by Southern hybridization. If the Tn*10* insertion is in *nrdD*, restriction fragment length polymorphisms between TT14888 and LT2 should be observed when a *nrdD* specific probe is used for hybridization. Two restriction enzymes digestion were employed. *Eco* RI can cut once at bp 3140 of Tn*10* (Way *et al.*, 1984) yield two restriction fragments. However, this enzyme cannot cut inside *nrdD*, therefore, from the *Eco* RI digested genomic DNA of LT2, only one hybridization signal of 6.5 kb was found. On the other hand, two hybridization signals, 7 kb and 9 kb, were found in *Eco* RI digested genomic DNA of TT14888 (TT14889). The change in restriction fragment length was due to insertion of Tn*10* in *nrdD* and therefore a new *Eco* RI site was created inside *nrdD*. So, the *nrdD* specific probe can detect two restriction fragments in TT14888 *Eco* RI-digested DNA. The total size of these two restriction fragments is 16 kb, therefore, a 9.5 kb difference was found between TT14888 and LT2. The difference is closed to the size of Tn*10* (9.3 kb). This can be further confirmed by using another enzyme *Pvu* I. This enzyme cannot cut Tn*10* but it can cut inside *nrdD*. From the nucleotide sequence of *nrdD*, two *Pvu* I cutting sites can be identified in the coding region of the gene.

Moreover, another *Pvu* I cutting site can be identified in the 5' untranslated region of the gene. Therefore, four restriction fragments should be identified. By calculation, the sizes of two of these fragments should be 1.6 kb, 1.7 kb, and the other two were unknown. However, the probe used can only detect three bands (two 2 kb and one 6 kb) as one *Pvu* I site at the most upstream location is beyond the probe, therefore, the restriction fragment upstream of this site cannot be identified. The sizes of hybridization signals can match those of the calculated fragments. The two 2 kb hybridization signals should be a duplex of 1.6 and 1.7 kb, and the size of one of the unknown fragments should be about 6 kb. However, the 1.6 kb fragment was missed from the TT14888/TT14889 *Pvu* I-digested DNA, instead, a novel 11 kb fragment was identified. So, Tn*10* should be inserted in the 1.6 kb fragment and it further confirmed that Tn*10* was inserted in *nrdD* in TT14888/TT14889. The restriction map and the Tn*10* insertion was illustrated in Fig. 5.5.

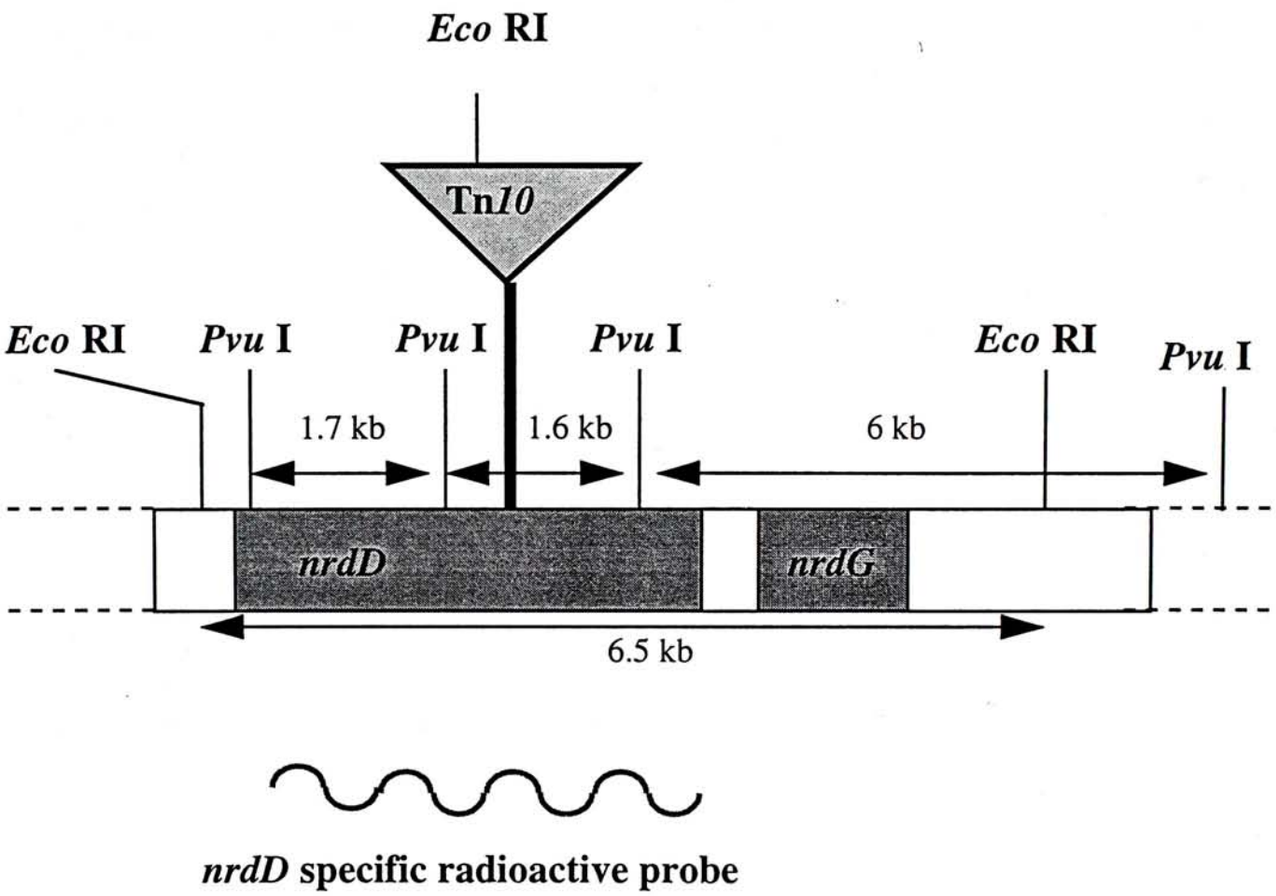

Fig. 5.5 Restriction map of *nrdDG operon* and position of Tn*10* insertion in TT14888. Diagram was not drawn in scale.

After confirming that *zzz*-3875::Tn*10* was located in *nrdD*, the effect of this mutation were compared in different genetic backgrounds. LT2 is the wild type strain which is $nrdA^+$ and $nrdD^+$. TT14888 is a mutant which is $nrdA_{ts}$ and $nrdD^-$. WL9701 is a transductant of P22 lysate of TT14888, its genotype should be $nrdA^+$ and $nrdD^-$. Apparently, there was no significant effect of *nrdD* mutation on aerobic growth of *S. typhimurium* since WL9701 showed no impaired growth in aerobic environment when compared to the wild type LT2. More surprisingly, *nrdD* mutation did not hinder the growth of WL9701 in anaerobic environment. Why WL9701 can grow normally in anaerobic environment? The answer could be inferred by observing the growth pattern of TT14888. At 30 and 37°C, TT14888 can grow normally in anaerobic environment. It means that the *nrdD* mutation did not harm the bacterial cells anaerobically at these temperatures. However, this mutant showed impaired growth when it was grown at 42°C anaerobically. The maximum OD600 of this mutant under this culture condition was 0.23 and change in biomass (OD600) was 0.16, 50% of the wild type. Therefore, it was the incubation temperature rather than the oxygen availability affecting the growth of TT14888 in anaerobic environment. Since TT14888 is a *nrdA* temperature sensitive mutant, i.e., NrdA is inactive at 42°C. This mutant showed very poor aerobic growth at 42 °C while no impaired growth was found in LT2 and WL9701. Hence, in 42 °C, both NrdA and NrdD of TT14888 could not function anaerobically, while WL9701 had a functional NrdA. Thus, the poor growth of TT14888 at this temperature was because that there was no functional NrdA to complement the lack of NrdD. On the other hand, under anaerobic growth at 42°C, WL9701 still had functional NrdA to complement the defective NrdD.

The same phenomenon was seen in *nrdD* and *nrdG* mutants of *E. coli* (Garriga *et al.*, 1996) and *Lactococcus lactis* (Jordan *et al.*, 1996b). These mutants can grow well in anaerobic conditions. While the effect of *nrdD* mutation in *E. coli* was found to be complemented by NrdAB, the effect of mutation was complemented by NrdEF in *L. lactis.* However, in *S. typhimurium*, NrdEF is cryptic in normal physiological state and it is not essential to the cell under both aerobic and anaerobic environments (Jordan *et al.*, 1996a), the possibility for NrdEF to take part in the complementation of *nrdD* mutation is low. But whether *nrdD* mutation will affect *nrdEF* expression still needed to be investigated.

Mutation of ribonucleotide reductase should be lethal since all four kinds of deoxyribonucleotides triphosphates (dNTPs) are synthesized from phosphorylation of corresponding deoxyribonucleotide diphosphate (dNDPs) by nucleotide diphosphate kinase. And dNDPs were synthesized by reduction of corresponding ribonucleotide diphosphates (NDPs) or triphosphates (NTPs) by ribonucleotide reductase (Reichard, 1988). Therefore, defects in ribonucleotide reductase would stop the *de novo* synthesis of dNDPs and dNTPs. However, TT14888 could grow at 42 °C. Both aerobic and anaerobic ribonucleotide reductases should be defective in this temperature, and the culture should not be able to grow. The surprising result was due to the dNTPs pool in the cells. Since the inoculum was from an aerobic culture at 37 °C, dNTPs production was normal in this situation. The dNTPs left inside the bacterial cell can support further growth of the bacteria though no *de novo* synthesis of dNTPs occurred. But the extent of growth was limited since the concentration of dNTPs would be used up after several divisions of bacterial cell. Therefore, the

anaerobic increase in biomass of TT14888 at 42 °C was limited and growth ceased at an early stage while the other two were still growing.

Chapter 6. General Discussions

6.1 General discussions

The genes encoding anaerobic ribonucleotide reductase and its activase had been cloned and sequenced from *Salmonella typhimurium* in this study. Anaerobic ribonucleotide reductase is encoded by *nrdD*, composed of an open reading frame of 2136 bp and an upstream promoter. In the promoter region of this gene, putative binding sequences for global regulator Fnr and ArcAB were identified. However, no consensus promoter sequence can be found. The amino acid sequence predicted from the nucleotide sequence of this gene shows striking homology of anaerobic ribonucleotide reductase of *E. coli*. Most of the functional domains of *E .coli* anaerobic ribonucleotide reductase are conserved in the *S. typhimurium* homologue. It seems that both enzymes behave similarly on the catalysis of reduction reaction. *S. typhimurium* NrdD also shows homology of anaerobic ribonucleotide reductases of other organisms such as *Haemophilus influenzae*, Bacteriophage T4, *Methanococcus jannaschii, Pyrococcus furiosus* and *Thermoplasma acidophilum.* Although these organisms are phylogenetically diversed, all of them possess anaerobic ribonucleotide reductases suggesting that this enzyme plays an indispensible role and conserved during the evolution process. Up to now, several microbial genomes had been completely sequenced, with the accumulation of information obtained from microbial genome project, much more anaerobic ribonucleotide reductases will be identified and the importance of ribonucleotide reductase may be inferred.

The gene encoding anaerobic ribonucleotide reductase activase, *nrdG*, was identified downstream of the *nrdD*. Two genes are separated by a short intervening

sequence and organized as an operon. The same organization was found in *E .coli.* Besides showing homology to the anaerobic ribonucleotide reductase activase, *nrdG* also show homology to another functionally different enzyme, pyruvate formate lyase activase (PflA). However, the similarity only confines to the catalytic domain of PflA. PflA uses radical chemistry to activate Pfl, hence, similar mechanism may be employed by NrdG to activate NrdD. This similarity can be arisen due to convergent evolution as well as divergent evolution. Further investigation is needed to confirm these two possibilities.

In *S. typhimurium*, the expression of *nrdD* is regulated by global regulator Fnr. The expression of *nrdD* increases 24-fold in anaerobic environment, but this increase was not found in a mutant defective in Fnr. It seems that Fnr acts as an activator of *nrdD* expression. A number of genes encoding enzymes important for anaerobic metabolism are found to be regulated by global transcriptional factors. It is possibly a strategy employed by enteric bacteria to adjust its metabolic context in order to adapt to the environment quickly. Since if each gene is only regulated by its own regulator, the response to the change in the environment is slow as numerous different regulatory proteins have to been synthesized. The expressions or repressions of this set of genes become synchronized if a global regulator is present to control them. The regulation also saves the resource of the bacterial cell by not producing unwanted or useless gene products. The study of regulation of anaerobic metabolism of enteric bacteria has mainly focused on the central metabolic pathway of the organism. This thesis provides evidences that enzymes important in anaerobic metabolism, other than those in the central metabolic pathway, are also regulated by global regulation.

Enteric bacteria possess at least two different sets of ribonucleotide reductases. One functions in aerobic environments and the other functions in anaerobic environments. This redundancy may have a physiological significance. From this study, a *S. typhimurium* mutant defective in NrdD can grow normally in anaerobic environments, while a mutant defective in both aerobic and anaerobic reductase show impaired anaerobic growth. It appears that the aerobic enzyme can complement the function of anaerobic enzyme. In *E. coli*, mutants defective in NrdD or NrdG cannot survive in strict anaerobic environments but both mutants can grow well in microaerophilic conditions. On the other hand, only conditional lethal mutant defective in *nrdAB* can be isolated suggests the function of aerobic ribonucleotide reductase cannot be substituted by NrdDG. Obligate anaerobes and facultative anaerobes differ from each other in their response to oxygen. One of the reasons that obligate anaerobes cannot survive in the presence of oxygen may due to lacking of a homologue of aerobic ribonucleotide reductase in these bacteria.

6.2 Further studies

More experiments have to be performed in order to fully understand the anaerobic ribonucleotide reductase of *S. typhimurium*.

1. To delineate the structure of the promoter of *nrdD* by primer extension analysis, S1 nuclease mapping or ribonuclease protection assay.
2. To investigate the properties of *S. typhimurium* NrdD such as cofactors used, substrate specificity and allosteric control by overexpressing of this enzyme.
3. To determine the expression level of *nrdG* by using *nrdDG* or *nrdG* specific probes.
4. To study the effect of *arcAB* on the *nrdD* and *nrdG* expression.
5. To study the effect of different electron acceptors such as nitrate, furmurate or TMAO on the expression of *nrdD* or *nrdG*.
6. To study the effect of these electron acceptors on the growth of *nrdD* defective mutant.
7. To test an absolute anaerobic culture condition for the growth of the *nrdD* defective mutant.

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